THE BIOCHEMICAL JOURNAL

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for phosphonofluoridate, *wherever mentioned* *read* phosphorofluoridate

Quantitative studies of the avidity of naturally occurring substances for trace metals. 3

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Volume 54 (1953), No. 4

p. 649, col. 2, line 5:
for $\log K_s = 36$ *read* $\log K_s = 32{\cdot}6$

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By F. B. Shorland

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p. 676, Table 3:
for but $\geqslant 0{\cdot}05$ *read* but $\leqslant 0{\cdot}05$

p. 676, Table 4:
for All differences not significant $P > 0{\cdot}5$ *read* All differences not significant $P > 0{\cdot}05$

Medical

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Action of Substituted Phenylalanines on *Escherichia coli*

By E. D. BERGMANN,* SARAH SICHER and B. E. VOLCANI
The Weizmann Institute of Science, Rehovoth, Israel

(*Received* 17 *June* 1952)

In a previous communication (Volcani, Sicher & Lichtenstein, 1951) it had been shown that *o*-hydroxyphenylalanine (OHPA) is an antagonist of phenylalanine and that it possesses different modes of action for different strains of *Escherichia coli*: it was inactive in a wild type and a tyrosineless mutant, but inhibited competitively a phenylalanineless mutant. It was concluded, therefore, that the compound interferes with the utilization, and not with the synthesis, of phenylalanine.

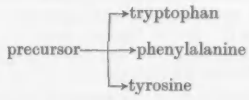
In the present investigation, other substituted phenylalanines were studied, both as to the point at which they block the pathway of the synthesis and utilization of the aromatic amino-acids and as to the nature of the influence of structural variations on the phenylalanine molecule, such as would permit graded changes in its chemical and physicochemical properties.

Particular interest attached to substituted phenylalanines because of their similarity to chloramphenicol and its active structural analogues (Bambas, Troutman & Long, 1950; Buu-Hoï, Hoán, Jacquignon & Khoï, 1950; Dann, Ulrich & Möller, 1950; Long & Troutman, 1951; Woolley, 1950; Truhaut, Lambin & Boyer, 1951).

The inhibition of *Esch. coli* by β-phenylserine which has previously been studied by Beerstecher & Shive (1946, 1947*a*) and Marnay (1951), has been shown by Woolley (1950) to apply also to the phenylalanineless mutant.

A recent paper by Maas & Davis (1950) and also the results recorded in the preceding communication (Volcani *et al.* 1951) have made it evident that when the antagonist-metabolite relation only is used in order to elucidate the point at which inhibition blocks biochemical reactions, the results are sometimes ambiguous. A clearer picture of the nature of the inhibition is obtained, as shown in the present study, by the additional use of mutants in which one particular mechanism is blocked genetically (Tatum, 1946).

For an evaluation of the results obtained by these two methods it is necessary to know the biogenetic relationships of the aromatic amino-acids. From the experiments of Beerstecher & Shive (1946, 1947*a*, *b*), the following 'straight' scheme suggests itself for the wild type of *Esch. coli*: precursor → tryptophan → phenylalanine → tyrosine → metabolic product of tyrosine. On the other hand, a 'branched' scheme of the type



though somewhat more complex, has been proposed by Davis (1950, 1951) for *Esch. coli* and by Atkinson, Melvin & Fox (1951) for *Lactobacillus arabinosus*. In this scheme, the three aromatic amino-acids are formed by parallel but independent routes from a common precursor. A decision between the two alternative schemes seemed possible by the study of 'sparing effects'; according to the straight scheme, and only according to it, phenylalanine and tyrosine would be expected to show a sparing effect in a tryptophanless mutant and tyrosine, but not tryptophan in a phenylalanineless mutant, whilst for a tyrosineless mutant neither tryptophan nor phenylalanine should show sparing effects. These effects have been observed in *Esch. coli*, although Simmonds, Tatum & Fruton (1947) and Simmonds (1950) have failed to detect them in a phenylalanineless and a tyrosineless mutant of the bacterium.

Inhibitors of various steps of the straight scheme have been discovered in *p*-nitrophenylalanine, *p*-fluorophenylalanine and 4-hydroxy-3-nitrophenylalanine (*m*-nitrotyrosine), which were tested on *Esch. coli*, wild type, and its tryptophan-, phenylalanine- and tyrosine-less mutants, as well as on a mutant which required all three aromatic amino-acids. Certain substituted β-phenylserines, whilst having no inhibitory action by themselves, counteract the inhibition caused by the phenylalanine derivatives. From the experimental results it is concluded that *Esch. coli* possesses the ability to circumvent the steps of the suggested straight pathway by alternative pathways, which are not normally utilized by the micro-organism. It has further been shown that, in the mutants inhibited by the above-mentioned phenylalanine derivatives, the sparing effect of the aromatic amino-acids for each other is more pronounced than without such inhibition.

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EXPERIMENTAL

Cultures and media. Cultures used included: *Esch. coli*, parent wild type strain (ATCC 9637), *Esch. coli* tryptophanless mutant (19–2), *Esch. coli* phenylalanineless mutant (M 83–5), *Esch. coli* tyrosineless mutant (M 83–9) and *Esch. coli* triply deficient mutant (M 83–2) (tryptophan + phenylalanine + tyrosine), all kindly supplied by Dr B. D. Davis. They were grown and assayed in the mineral medium of Davis & Mingioli (1950) as described previously. Media and experimental procedures were those employed in a preceding paper (Volcani *et al.* 1951). All experiments were carried out in duplicate.

For inoculum in the 'sparing' experiments, the surface growth from an 8 hr. culture was gently scraped off and suspended in sterile 0·9% NaCl solution to a turbidity reading of 56–60 on the Klett-Summerson photoelectric colorimeter using a no. 64 red filter. From this heavy suspension, 0·3 ml. was diluted into 10 ml. sterile saline; each experimental culture was inoculated with a drop of this dilute suspension. Final volume 5 ml. Cultures were incubated at 37° for 18 hr. and growth turbidity was determined as above.

In the inhibitor experiments, DL-*p*-aminophenylalanine (PAPA), DL-*p*-nitrophenylalanine (PNPA), L-4-hydroxy-3-nitrophenylalanine (MNT), *o*-hydroxyphenylalanine (OHPA), DL-β-phenylserine (PS), and DL-tryptophan, DL-phenylalanine and L-tyrosine were added to a series of test tubes containing 2·5 ml. of the double-strength medium. The cultures were diluted to 5 ml. with distilled water, and autoclaved at 15 lb. pressure for 15 min. DL-*p*-Fluorophenylalanine (PFPA), and the substituted β-phenylserines were added aseptically as aqueous solutions which had been filtered through a glass filter. The final volume used was 2·5 ml.

Materials. DL-*p*-Nitro-β-phenylalanine was prepared according to Erlenmeyer & Lipp (1883), DL-*p*-amino-β-phenylalanine according to Bergmann (1952). The former was recrystallized from *tert.*-butanol, m.p. 240–245° (decomp.) (Burckhalter & Stephens (1951): 222–240°), the latter from boiling water, m.p. 234° (decomp.) (after drying at 140° *in vacuo*) (Burckhalter & Stephens (1951): 235–242°).

DL-*p*-Fluoro-β-phenylalanine, which was obtained by the method of Bergmann (1952), crystallized from a mixture of water and ethanol (3:1) in needles of m.p. 238–242° (decomp.).

For the synthesis of L-4-hydroxy-3-nitro-β-phenylalanine, from water or glacial acetic acid, m.p. 225–230° (decomp.), the nitration of L-tyrosine (Staedeler, 1860; Johnson & Kohmann, 1915), was employed, and for the preparation of DL-β-phenylserine (*threo*) the method of the Gesellschaft für Kohlentechnik (1936) and of Carrara & Weitnauer (1949); from 50% ethanol, m.p. 196° (decomp.).

DL-*p*-Nitro-β-phenylserine, from water, m.p. 186° (decomp.), has been obtained according to Dalgliesh (1949) by hydrolysis of its esters (Bergmann, Genas & Bendas, 1950; Bergmann, Bendas & Taub, 1951); its hydrogenation (Dalgliesh, 1949) gave DL-*p*-amino-β-phenylserine, from water, m.p. 220° (decomp.).

DL-*m*-Nitro-β-phenylserine, from water, m.p. 208–210°, has been described before by Bergmann *et al.* (1950, 1951).

DL-m-*Amino-β-phenylserine.* In the manner prescribed for the *p*-compound (Dalgliesh, 1949), DL-*m*-nitro-β-phenylserine (1·82 g.) was hydrogenated in aqueous medium. The filtered solution was evaporated to dryness *in vacuo* and the residue treated with aqueous ammonia solution and a little ethanol. Upon standing, the desired *product* separated. It was recrystallized from *tert.*-butanol or *iso*propanol, containing a little water; m.p. 154–157° (decomp.). (Found: C, 55·0; H, 6·1; N, 14·4. $C_9H_{12}O_3N_2$ requires C, 55·1; H, 6·1; N, 14·3%.)

RESULTS

Sparing experiments

Tryptophanless mutant

Response to DL-*phenylalanine.* Whilst the tryptophanless mutant failed to grow on phenylalanine alone, addition of increasing amounts of DL-phenylalanine to various levels of tryptophan caused an increase in growth; the combination of 12 μg./ml. of DL-tryptophan with 40 μg./ml. of DL-phenylalanine gave full growth (Fig. 1).

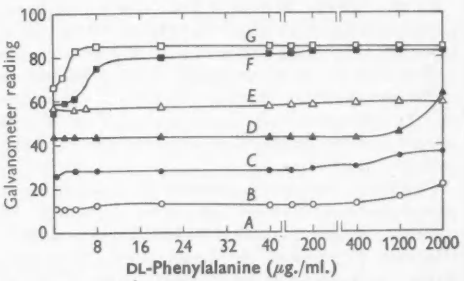


Fig. 1. Sparing effect of phenylalanine on tryptophan requirements of *Esch. coli*, tryptophanless mutant, at various tryptophan levels. Curve *A*, no tryptophan. Curves *B–G*, 0·4, 1, 2, 4, 12 and 20 μg./ml. tryptophan, respectively.

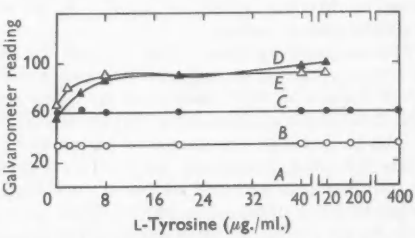


Fig. 2. Sparing effect of tyrosine on tryptophan requirements of *Esch. coli*, tryptophanless mutant, at various tryptophan levels. Curve *A*, no tryptophan. Curves *B–E*, 1, 4, 12 and 20 μg./ml. tryptophan, respectively.

Response to L-*tyrosine.* Effects are observed analogous to those with DL-phenylalanine. At the level of 12 μg./ml. of DL-tryptophan, the addition of 20 μg./ml. of L-tyrosine caused the growth to exceed the maximum growth possible with tryptophan alone (Fig. 2).

Phenylalanineless mutant

Response to DL-*tryptophan*. The phenylalanineless mutant could not utilize tryptophan in any concentrations up to 1000 μg./ml. Addition of increasing amounts of DL-tryptophan to various levels of DL-phenylalanine had only a negligible effect. At initial growth levels of 38 and 50 %, 600 μg./ml. of tryptophan caused a 7 % increase in growth.

Response to L-*tyrosine*. As shown in Fig. 3, the phenylalanineless mutant failed to grow in the presence of tyrosine alone (from 0 to 1000 μg./ml.). Addition of increasing amounts of L-tyrosine to various levels of phenylalanine, however, caused appreciable increases in growth. The sparing effect of tyrosine was most marked around an initial growth of 20 %, and it decreased with ascending growth levels, showing no effect above 70 % of full growth. At none of the phenylalanine concentrations studied, did addition of tyrosine cause full growth.

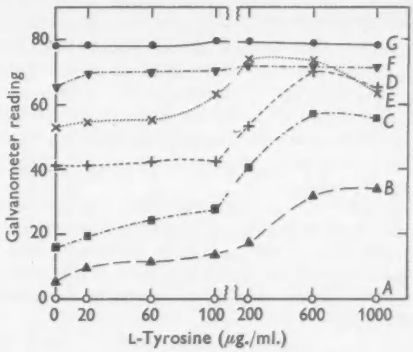


Fig. 3. Sparing effect of tyrosine on phenylalanine requirements of *Esch. coli*, phenylalanineless mutant, at various phenylalanine levels. Curve *A*, no phenylalanine. Curves *B–G*, 1, 2, 4, 10, 14 and 18 μg./ml. phenylalanine, respectively.

Table 1. *Reversal, by the amino-acids* DL-*tryptophan*, DL-*phenylalanine and* L-*tyrosine, of the inhibition of* Esch. coli, *wild type, by various substituted phenylalanines*

(Full growth gives galvanometer reading of 98. Antibacterial index = (μg. inhibitor/ml.)/(μg. amino-acid/ml.).)

Inhibitor (μg./ml.)	Amino-acid	Quantity required for 50% growth (galv. reading: 49) (μg./ml.)	Anti-bacterial index
A. DL-*p*-Aminophenylalanine (PAPA)			
	Galv. reading without amino-acids for 1 μg./ml. PAPA: 49 for 4 μg./ml. PAPA: 0		
6	Tyrosine	0·18	33
10	Tyrosine	0·32	31
20	Tyrosine	0·50	40
6	Phenylalanine	0·72	7·6
10	Phenylalanine	1·30	7·7
20	Phenylalanine	2·70	7·4
6	Tryptophan	2·40	2·5
10	Tryptophan	4·80	2·1
20	Tryptophan	40·0	0·5
B. DL-*p*-Fluorophenylalanine (PFPA)			
	Galv. reading without amino-acids for 3 μg./ml. PFPA: 49 for 60 μg./ml. PFPA: 0		
4	Tyrosine	0·6	6·6
40	Tyrosine	3·0	13·3
4	Phenylalanine	0·7	8·0
40	Phenylalanine	3·9	10·2
4	Tryptophan	1·4	2·8
40	Tryptophan	*	—
C. DL-*m*-Nitrotyrosine (MNT)			
	Galv. reading without amino-acids for 120 μg./ml. MNT: 49 for 400 μg./ml. MNT: 0		
200	Tyrosine	1·4	143
600	Tyrosine	9·4	64
200	Phenylalanine	6	33
600	Phenylalanine	460	1·3
200	Tryptophan	0·7	285
600	Tryptophan	0·9	666
D. β-Phenylserine (PS)			
	Galv. reading without amino-acids for 560 μg./ml. PS: 49 for 1000 μg./ml. PS: 0		
600	Tyrosine	2500	0·2
1000	Tyrosine	1111	0·9
600	Phenylalanine	3000	0·2
1000	Phenylalanine	833	1·2
600	Tryptophan	1500	0·4
1000	Tryptophan	625	1·6

SUMMARY

Ratio antibact. index tyrosine:2 × antibact. index phenylalanine:2 × antibact. index tryptophan.† (Antibact. index tyrosine = 1.)

Inhibitor	(μg./ml.)	Ratio	Inhibitor	(μg./ml.)	Ratio
PAPA	6	1:2·1:6·6	MNT	200	1:2·1:0·25
	10	1:2·0:7·5		600	1:24·4:0·05
	20	1:2·6:40·0	PS	600	1:0·5:0·6
PFPA	4	1:0·4:1·1		1000	1:0·75:0·56
	40	1:0·6:—			

* 200 μg./ml. tryptophan restored growth to 20 % (galvanometer reading 20). Higher concentrations inhibited growth, fully at 2000 μg./ml.

† Ratio calculated on the basis of the L-isomer.

Tyrosineless mutant

Response to DL-*tryptophan*. The tyrosineless mutant did not utilize tryptophan alone in any concentration up to 1000 μg./ml. Addition of graded amounts of DL-tryptophan (up to 1000 μg./ml.) at various levels of L-tyrosine (yielding 20 and 50 % of full growth) showed no effect on the growth of the organism.

Response to DL-*phenylalanine*. Phenylalanine was not utilized in concentrations up to 1000 μg./ml. The addition of 600 μg./ml. at different levels of tyrosine (giving 16, 25, 52 and 90 % of full growth) caused increases of 5–12 % at the lower growth levels only.

Inhibition experiments

Response of Esch. coli, *wild type*

The amounts of each of the phenylalanine derivatives and of β-phenylserine needed to reduce growth of *Esch. coli*, wild type, to 50 % of the maximum, or to cause complete inhibition, were determined from a dose-response curve. The reversal of growth inhibition by tryptophan, phenylalanine and tyrosine was tested at various inhibitory levels, and the antagonist ratio obtained from the corresponding growth curves. The antagonist ratios of the inhibiting compounds to the respective amino-acids ('antibacterial index') which permit half maximum growth, and the ratios of the concentrations of the aromatic amino-acids which also give 50 % growth, are summarized in Table 1.

p-Aminophenylalanine (PAPA) inhibits the wild strain of *Esch. coli*. All three aromatic amino-acids reverse the inhibition, but the amounts required for this purpose vary significantly, tyrosine being the most, tryptophan the least effective. This ratio points to the utilization of tyrosine as the step which is blocked by the inhibitor. In the case of *p*-fluorophenylalanine (PFPA) and *m*-nitrotyrosine (MNT), which also have an inhibiting effect (PFPA of approximately the same order of magnitude as PAPA, MNT very much less), the ratio of the reversing activities of the three aromatic amino-acids is different; in the former case, phenylalanine, in the latter tryptophan proved the most effective. PFPA, undoubtedly, interferes with the utilization of phenylalanine, whilst MNT appears to block the synthesis of tryptophan. However, it will be shown later that this is not the only effect exerted by the anti-metabolite. Phenylserine (PS) also shows a significant inhibiting action only in concentrations of 500–1000 μg./ml., and in the reversal of this effect, phenylalanine seems to be the most active of the three aromatic amino-acids.

None of the compounds *p*-nitrophenylalanine (PNPA), *p*-amino-β-phenylserine (PAPS), *p*-nitro-β-phenylserine (PNPS), *m*-amino-β-phenylserine (MAPS) and *m*-nitro-β-phenylserine (MNPS) affected growth, when added in quantities from 0 to 2000 μg./ml.

In this connexion it seemed interesting to test the claim of Beerstecher & Shive (1947*b*) that arginine, glutamic acid and cystine are precursors of a metabolite which is involved in the synthesis of phenylalanine. L-Arginine hydrochloride, L-glutamic acid and L-cystine, added in quantities up to 100 μg./ml. to an inhibiting concentration of 10 μg./ml. of *p*-aminophenylalanine, were completely inactive.

Response of the tryptophanless mutant

The action of PAPA, PFPA and β-phenylserine (PS) on this mutant and the antagonist:tryptophan ratios are summarized in Table 2. PFPA had a much stronger inhibiting effect than PAPA, and the inhibiting action of PS is weak. The other substances investigated showed no effect on the tryptophanless mutant.

Table 2. *Inhibition of* Esch. coli, *tryptophanless mutant, by substituted phenylalanines, and its reversal by* DL-*tryptophan*

(Maximum growth, with tryptophan alone, gives galv. reading of 85. Antibacterial index = (μg. inhibitor/ml.)/(μg. amino-acid/ml.).)

Inhibitor	(μg./ml.)	Tryptophan (μg./ml.)	Galvanometer reading	Antibacterial index at galv. reading 42·5 (50 % of full growth)
None	—	1·2	28	—
PAPA	20	1·2	14	—
	16	3·2	42·5	5
	40	20	42·5	2
	400	No reversion	—	—
PFPA	4	1·2	14	—
	4	40	42·5	0·1
	20	No reversion	—	—
PS	1200	1·2	14	—
	400	1·0	42·5	400
	1200	3·0	42·5	400
	2000	10·0	42·5	200

Response of the phenylalanineless mutant

The mutant could not utilize *p*-aminophenylalanine (PAPA) in amounts up to 600 μg./ml. instead of phenylalanine.

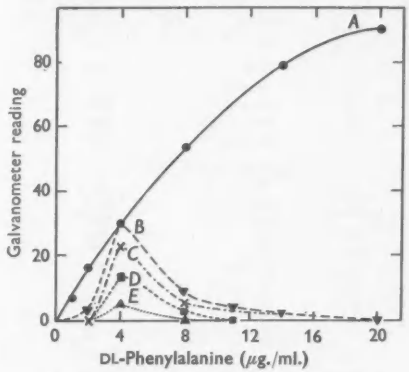


Fig. 4. Effect of phenylalanine and *p*-aminophenylalanine (PAPA) on growth of *Esch. coli*, phenylalanineless mutant. Curve *A*, phenylalanine alone. Curves *B–E* obtained with 400, 600, 800 and 1000 μg./ml. PAPA, respectively. Incubated 18 hr. at 37°.

Fig. 4 shows the effect of increasing amounts of DL-phenylalanine on various inhibiting levels of PAPA. At 400 μg./ml. PAPA, 2 μg./ml. of phenylalanine restored growth to the level caused by phenylalanine alone. Above that concentration, phenylalanine was synergic in increasing the inhibition. This effect was enhanced with increasing concentrations of phenylalanine. For inhibiting concentrations of PAPA, PFPA, and PS and the antagonist:phenylalanine ratios see Table 3. No effect was observed when *p*-nitrophenylalanine (PNPA) and *m*-nitrotyrosine (MNT) were added at 30% of full growth (4 μg./ml. DL-phenylalanine).

Response of the tyrosineless mutant

The actions of PAPA, PNPA, PFPA and MNT are summarized in Table 4. The organism could not effectively utilize PAPA (up to 2000 μg./ml.) instead of tyrosine. In the presence of tyrosine, however, growth of the mutant was enhanced by low concentrations of PAPA, while higher ones caused inhibition.

In contradistinction to *Esch. coli*, wild type, and its phenylalanineless mutant, the growth of the tyrosineless mutant was stimulated by low concentrations, and inhibited by high concentrations, of PNPA. The mutant could not, however, utilize effectively PNPA (up to 800 μg./ml.) alone in place of tyrosine.

It should be noted that for equal inhibition, much larger quantities of PAPA are required by the tyrosineless mutant than by the phenylalanineless one, the tryptophanless one and the wild type. This fact gives a lead as to the point of attack of the inhibitor.

None of the four β-phenylserine derivatives affected growth of the mutant.

Response of the triply deficient mutant

In the triply deficient mutant strain M 83–2 the relationship between the individual aromatic amino-acids and the antagonists could be studied

Table 3. *Inhibition of* Esch. coli, *phenylalanineless mutant, by substituted phenylalanines, and its reversal by* DL-*phenylalanine*

(Maximum growth, with phenylalanine alone, gives galv. reading of 92. Antibacterial index = (μg. inhibitor/ml.)/(μg. amino-acid/ml.).)

Inhibitor	(μg./ml.)	Phenylalanine (μg./ml.)	Galvanometer reading	Antibacterial index at galv. reading 46 (50% of full growth)
None	—	2	17	—
	—	4	24	—
	—	6	46	—
	—	8	51	—
PAPA	110	8	26	—
	600	8	0	—
	400	3·0	46	133
	600	3·2	46	187
	800	3·2	46	250
	1000	3·2	46	312
PFPA	36	6	23	—
	1000	6	0	—
	60	9·6	46	6·3
	100	16·0	46	6·3
	200	25·6	46	7·8
PS	600	4	12	—
	600	11·0	46	55
	2000	13·0	46	150

Table 4. *Inhibition of* Esch. coli *tyrosineless mutant, by substituted phenylalanines, and its reversal by* L-*tyrosine*

(Maximum growth, with tyrosine alone, gives galv. reading of 92. Antibacterial index = (μg. inhibitor/ml.)/(μg. amino-acid/ml.).)

Inhibitor	(μg./ml.)	Tyrosine (μg./ml.)	Galvanometer reading	Antibacterial index at galv. reading 46 (50% of full growth)
None	—	2	23	—
PAPA	900	2	11·5	—
	1600	2	0	—
	1000	3·8	46	263
	1600	9·8	46	163
	2000	11·6	46	172
PNPA	1300	2	11·5	—
	2000	2	0	—
	1000	3·2	46	312
	2000	5·6	46	357
	4000	12·5	46	320
MNT	490	2	11·5	—
	600	2	0	—
	600	6·2	46	97
	1000	52·0	46	17
	1600	480·0	46	3·5

more specifically, by keeping constant two of the required acids and varying the third together with the given antagonist.

p-*Aminophenylalanine*. Low concentrations of PAPA decrease only slightly the growth obtained with 20 μg./ml. each of DL-tryptophan and DL-phenylalanine and 1 μg./ml. of L-tyrosine. The effect becomes marked above 400 μg./ml. PAPA and inhibition is complete at 2000 μg./ml. of the antagonist; the inhibition is reversed by tyrosine (at 2000 μg./ml. PAPA by 40 μg./ml.).

When the organism is grown in the presence of 20 μg./ml. each of DL-tryptophan and L-tyrosine and of 2 μg./ml. of DL-phenylalanine, the inhibiting effect of PAPA becomes marked at 120 μg./ml. and is complete at 200 μg./ml. Relatively small doses of phenylalanine (8 μg./ml. at 120 μg./ml. PAPA) bring the level back to about 70% of full growth; for complete reversal much larger quantities (40 μg./ml.) of phenylalanine are necessary.

p-*Fluorophenylalanine*. In the presence of 20 μg./ml. of DL-tryptophan, 20 μg./ml. L-tyrosine and 2 μg./ml. DL-phenylalanine, already low concentrations of PFPA (8 μg./ml.) bring about complete inhibition. This is fully reversed by 20 μg./ml. of phenylalanine. As in the phenylalanineless mutant, the antagonism is competitive and indicates, thus, that the inhibitor affects the utilization of phenylalanine.

o-*Hydroxyphenylalanine*. This compound was about half as active an inhibitor of PFPA under the same conditions of growth.

m-*Nitrotyrosine*. Whilst 120 μg./ml. of MNT caused a 50% reduction in growth rate in the presence of 20 μg./ml. of tryptophan, 20 μg./ml. of phenylalanine and 1 μg./ml. of tyrosine, for 75 and 100% inhibition, 400 and 1200 μg./ml., respectively, were required. This inhibition is reversed by L-tyrosine. The behaviour of the triply deficient mutant towards *m*-nitrotyrosine thus resembles that of the tyrosineless mutant of *Esch. coli*.

Reversal of PAPA, PFPA, MNT and PS inhibition of Esch. coli, *wild type, by PAPS, PNPS, MAPS and MNPS*

The various compounds (PAPS, PNPS, MAPS and MNPS) were added in amounts varying from 0 to 1000 μg./ml. to the basal medium, containing quantities of the antagonists which gave inhibition levels of 40–70% ('lower') and 90–100% ('higher'), respectively. Growth response curves were drawn; the maximal effective quantities of 'the anti-antagonist' that reversed the inhibition at both levels are summarized in Table 5.

It was shown that both *m*-amino- and *m*-nitrophenylserine counteract the inhibition of the wild type *Esch. coli*, which is caused by PAPA, PFPA, MNT and PS, whilst *p*-aminophenylserine was less effective and the corresponding *p*-nitro compound entirely devoid of any activity.

In a number of systems studied, involving the inhibited phenylalanineless and tyrosineless mutants of *Esch. coli*, practically no reversal of the above type was observed.

Table 6, which summarizes the experimental results and includes also the previously (Volcani *et al.* 1951) reported data for *o*-hydroxyphenylalanine, illustrates the fact that a seemingly slight variation in the structure of the substituted phenylalanines investigated, changes the point of attack of these compounds; it gives examples of almost every possible combination of effects.

Table 5. *Comparative effectiveness of* p-*aminophenylserine* (*PAPS*) p-*nitrophenylserine* (*PNPS*), m-*aminophenylserine* (*MAPS*) *and* m-*nitrophenylserine* (*MNPS*), *in overcoming the inhibition of* Esch. coli, *wild type, by* p-*aminophenylalanine* (*PAPA*), p-*fluorophenylalanine* (*PFPA*), m-*nitrotyrosine* (*MNT*) *and* β-*phenylserine* (*PS*)

A1. PAPS *v.* PAPA

PAPA (μg./ml.)	PAPS (μg./ml.)	Galv. reading
8	—	60
60	—	16
8	600	72
8	1000	65
60	200–600	22
60	1000	16

A2. PNPS *v.* PAPA

PAPA (μg./ml.)	PNPS (μg./ml.)	Galv. reading
8	—	53
60	—	15
8	200–1000	60
60	0–1000	15

A3. MAPS *v.* PAPA

PAPA (μg./ml.)	MAPS (μg./ml.)	Galv. reading
8	—	50
40	—	12
8	60	81
8	100	86
40	1000	70

A4. MNPS *v.* PAPA

PAPA (μg./ml.)	MNPS (μg./ml.)	Galv. reading
10	—	46
40	—	18
10	1000	91
40	1000	40

B1 PAPS *v.* PFPA

PFPA (μg./ml.)	PAPS (μg./ml.)	Galv. reading
4	—	34
40	—	3
4	1000	86
40	0–1000	3

B2. PNPS *v.* PFPA

PFPA (μg./ml.)	PNPS (μg./ml.)	Galv. reading
4	—	36
40	—	0
4	0–1000	36
40	0–1000	6

B3. MAPS *v.* PFPA

PFPA (μg./ml.)	MAPS (μg./ml.)	Galv. reading
4	—	43
40	—	0
4	1000	88
40	60–1000	10–14

B4. MNPS *v.* PFPA

PFPA (μg./ml.)	MNPS (μg./ml.)	Galv. reading
4	—	42
40	—	0
4	1000	90
40	0–1000	0

C1 PAPS *v.* MNT

MNT (μg./ml.)	PAPS (μg./ml.)	Galv. reading
100	—	58
200	—	10
100	1000	72
200	10–1000	16

C2. PNPS *v.* MNT

MNT (μg./ml.)	PNPS (μg./ml.)	Galv. reading
100	—	53
200	—	7
100	1000	7
200	0–1000	7

C3. MAPS *v.* MNT

MNT (μg./ml.)	MAPS (μg./ml.)	Galv. reading
100	—	53
200	—	7
100	1000	92
200	1000	83

C4. MNPS *v.* MNT

MNT (μg./ml.)	MNPS (μg./ml.)	Galv. reading
100	—	56
200	—	9
100	1000	85
200	1000	62

D1. PAPS *v.* PS

PS (μg./ml.)	PAPS (μg./ml.)	Galv. reading
600	—	43
1000	—	0
600	600–1000	90
1000	1000	25

D2. PNPS *v.* PS

PS (μg./ml.)	PNPS (μg./ml.)	Galv. reading
600	—	37
1000	—	0
600	0–1000	37
1000	0–1000	0

D3. MAPS *v.* PS

PS (μg./ml.)	MAPS (μg./ml.)	Galv. reading
600	—	33
1000	—	0
600	600–1000	91
1000	0–40	0
1000	1000	90

D4. MNPS *v.* PS

PS (μg./ml.)	MNPS (μg./ml.)	Galv. reading
600	—	32
1000	—	0
600	200–1000	98
1000	0–190	0
1000	600–1000	84

Table 6. *Effect of various substituted phenylalanines and phenylserine on growth of* Esch. coli *and its mutants*

Compound	Wild type	Trypto-phanless mutant	Phenyl-alanineless mutant	Tyrosineless mutant	Triply deficient mutant: Tryptophan	Triply deficient mutant: Phenyl-alanine	Triply deficient mutant: Tyrosine
p-Aminophenylalanine (PAPA)	+	+	+*	+	+	+	+
p-Nitrophenylalanine (PNPA)	–	–	+*	+	–	–	+
p-Fluorophenylalanine (PFPA)	+	+	+	–	+	+	–
m-Nitrotyrosine (MNT)	+	–	–	+	–	–	+
o-Hydroxyphenylalanine (OHPA)	–	–	+	–	–	+	–
β-Phenylserine (PS)	+	+	+	–	+	+	–

–, no effect; +, inhibition; * growth enhancement at low concentrations of the compound in the presence of tyrosine.

'*Sparing effects*' *in the inhibited mutants*†

Tryptophanless mutant, inhibited by p-*aminophenylalanine* (*PAPA*)

Sparing effect of phenylalanine on tryptophan. The organism was grown in the presence of 4 μg./ml. of tryptophan and inhibited by 40 and 200 μg./ml., respectively, of PAPA (70 and 100% inhibition, respectively). 20 and 200 μg./ml., respectively, of phenylalanine reversed the inhibition to 75% of full growth.

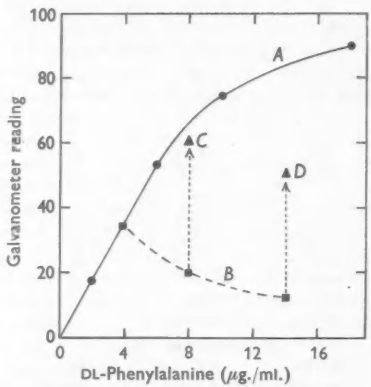


Fig. 5. Reversal of *p*-aminophenylalanine-inhibited phenylalanineless *Esch. coli* by DL-tryptophan. Curve *A*, growth in the presence of phenylalanine. Curve *B*, growth in the presence of phenylalanine and 400 μg./ml. PAPA. Curve *C*, addition of 100–600 μg./ml. of tryptophan to 400 μg./ml. PAPA at 8 μg./ml. of phenylalanine. Curve *D*, addition of 600 μg./ml. of tryptophan to 400 μg./ml. PAPA at 14 μg./ml. of phenylalanine. Incubated 18 hr. at 37°.

† Percentages of growth given in this section relate to maximum growth obtained with designated quantities of substrates.

Sparing effect of tyrosine on tryptophan. When the organism was grown under the same conditions as in the previous paragraph, addition of 4 μg./ml. of tyrosine at 80, and of 8 μg./ml. of tyrosine at 200 μg./ml. PAPA restored 75% of the full growth.

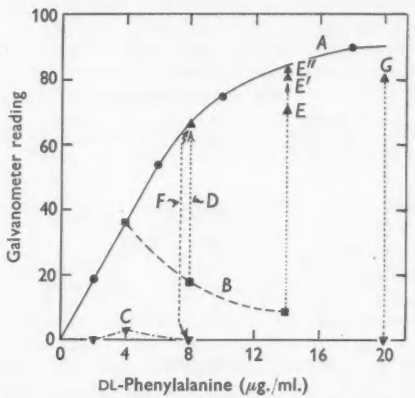


Fig. 6. Reversal of *p*-aminophenylalanine-inhibited phenylalanineless *Esch. coli* by L-tyrosine. Curve *A*, growth in the presence of phenylalanine. Curves *B* and *C*, growth in the presence of phenylalanine with 400 and 1000 μg./ml. PAPA, respectively. Curve *D*, addition of 8 μg./ml. of tyrosine to 400 μg./ml. PAPA at 8 μg./ml. of phenylalanine. Curves *E*, *E'* and *E''*, addition of 1, 2 and 20 μg./ml. of tyrosine, respectively, to 400 μg./ml. PAPA at 14 μg./ml. of phenylalanine. Curve *F*, addition of 0·6 μg./ml. of tyrosine to 1000 μg./ml. PAPA at 8 μg./ml. of phenylalanine. Curve *G*, addition of 6 μg./ml. tyrosine to 1000 μg./ml. PAPA at 20 μg./ml. of phenylalanine. Incubated 18 hr. at 37°.

Phenylalanineless mutant, inhibited by p-*aminophenylalanine* (*PAPA*)

Sparing effect of tryptophan on phenylalanine. In the presence of 8 and 14 μg./ml. phenylalanine, addition of 400 μg./ml. PAPA decreased the growth

by 70 and 86 %, respectively. Addition of increasing amounts of DL-tryptophan gradually reversed the inhibition to 90 and 60 %, respectively, of full growth. The effects are schematically presented in Fig. 5.

Sparing effect of L-*tyrosine on phenylalanine.* The mutant was grown in the presence of 8 and 14 μg./ml. phenylalanine and 400 μg./ml. PAPA, which permitted only 10 and 27 % growth, respectively. When increasing amounts of L-tyrosine were added at these two levels of inhibition, growth was restored from 27 to 100 % by 8 μg./ml. tyrosine, and from 10 to 95 % by 2 μg./ml. tyrosine. Fig. 6 summarizes all the data obtained.

Tyrosineless mutant, inhibited by PAPA

Sparing effect of tryptophan on tyrosine. In the presence of 8 and 12 μg./ml. tyrosine and 1600 μg./ml. PAPA, DL-tryptophan, added in a wide range of concentrations (up to 1000 μg./ml.), was without effect.

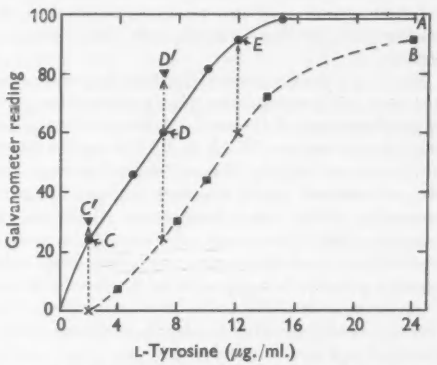


Fig. 7. Reversal of *p*-aminophenylalanine-inhibited tyrosineless *Esch. coli* by DL-phenylalanine. Curve *A*, growth in the presence of tyrosine. Curve *B*, growth in the presence of tyrosine with 1600 μg./ml. PAPA. Curves *C* and *C'*, addition of 12 and 20 μg./ml. of phenylalanine, respectively, to 1600 μg./ml. PAPA at 2 μg./ml. of tyrosine. Curves *D* and *D'*, addition of 0·8 and 2–20 μg./ml. of phenylalanine, respectively, to 1600 μg./ml. PAPA at 7 μg./ml. of tyrosine. Curve *E* addition of 0·6–20 μg./ml. of phenylalanine to 1600 μg./ml. PAPA at 12 μg./ml. of tyrosine. Incubated 18 hr. at 37°.

Sparing effect of phenylalanine on tyrosine. The organism was grown in the presence of 2, 7 and 12 μg./ml. tyrosine and inhibited with 1600 μg./ml. PAPA. As schematically shown in Fig. 7, the inhibition was completely reversed by phenylalanine in all cases, the quantities required being 12, 0·8 and 0·6 μg./ml. respectively. Addition of phenylalanine above the concentrations required for complete reversal increased the growth response at low inhibiting levels still further. The effect is most noticeable at 7 μg./ml. tyrosine, where addition of 2 μg./ml. phenylalanine stimulated growth by 20 % above maximum growth at that tyrosine level—a level which could be reached only by addition of 10 μg./ml. tyrosine.

Other inhibitors

In view of the above results, it seemed interesting to study the effect of known inhibitors on the utilization of phenylalanine and tyrosine by the tryptophanless mutant, of tryptophan and tyrosine by the phenylalanineless mutant, and of phenylalanine by the tyrosineless mutant. Experiments with the latter mutant and tryptophan were not carried out, as this amino-acid had proved inactive in the above experiments with PAPA.

Effect of p-*fluorophenylalanine (PFPA) on the utilization of phenylalanine and tyrosine by the tryptophanless mutant.* Both at 10 % of full growth and complete inhibition (8 and 20 μg./ml. of PFPA, in presence of 4 μg./ml. of tryptophan), DL-phenylalanine in quantities of 4 and 8 μg./ml. respectively, gives 85 % of full growth. Even quantities of 2000 μg./ml. do not restore full growth. Very similar effects were observed in parallel experiments with L-tyrosine: 2 and 20 μg./ml. of tyrosine restored full growth at the two levels, respectively.

Effect of PFPA on the utilization of tryptophan and tyrosine by the phenylalanineless mutant. When the growth of the mutant (with 8 μg./ml. phenylalanine) was reduced to 35 % of full growth by 80 μg./ml. PFPA, addition of 200 and 600 μg./ml. of DL-tryptophan increased growth very slightly, i.e. by 7 and 12 %, respectively (as compared to 3 and 7 % increase obtained with tryptophan alone in the above concentrations). Addition of up to 60 μg./ml. tyrosine showed no effect on the growth of the organism; 100–200 μg./ml. tyrosine increased growth by 9 %; at 600 μg./ml. this effect dropped slightly (to 5 %).

Effect of β-phenylserine (PS) on the utilization of tryptophan and tyrosine by the phenylalanineless mutant. In these experiments, too, at 35 % of full growth (10 μg./ml. phenylalanine and 1000 μg./ml. PS), addition of DL-tryptophan or L-tyrosine in a wide range of concentrations (up to 600 μg./ml.), did not affect the growth of the organism.

Effect of m-*nitrotyrosine (MNT) on the utilization of phenylalanine by the tyrosineless mutant.* In the presence of 8 μg./ml. L-tyrosine and 600 μg./ml. MNT (52 % of full growth), addition of increasing amounts of DL-phenylalanine (up to 100 μg./ml.) caused no response; at phenylalanine levels of 200–1000 μg./ml., a 10 % increase of growth was observed. Similar stimulation is caused by 60–1000 μg./ml. phenylalanine in the uninhibited mutant.

DISCUSSION

In the tyrosineless mutant, the addition of tryptophan was without effect, and that of phenylalanine was practically negligible. In the phenylalanineless mutant, however, a sparing effect of tyrosine, and in the tryoptophanless mutant a pronounced synergism, if not a sparing effect of both phenylalanine and tyrosine has been demonstrated. It may be emphasized that these effects are evident only under certain well-specified conditions, and this may be the reason why Simmonds *et al.* (1947) failed to observe them in their experiments. They are compatible only with a straight scheme, not, at least for the mutants used, with a branched one (see p. 1). In this connexion, it may be recalled that in higher animals the conversion of phenylalanine to tyrosine has been demonstrated beyond doubt (Moss & Schoenheimer, 1940; Womack & Rose, 1946; Jervis, 1947; Grau, 1947; Lerner, 1949; Udenfriend, Cooper & Clark, 1951).

It may well be possible that, in general, the relationship between the aromatic amino-acids cannot be described by a single scheme. The following discussion, however, is based on the straight scheme; it is assumed that a compound which inhibits the 'A'-requiring mutant in a competitive manner, does so because it affects the utilization of the metabolite 'A'.

p-*Aminophenylalanine* (*PAPA*). PAPA inhibits competitively the utilization of tyrosine; replacement of the hydroxyl group of tyrosine by the amino group evidently brings about an increased affinity of the phenylalanine molecule for the enzyme concerned. One may also conclude that tryptophan and phenylalanine reverse the effect, in the wild strain, due to their conversion into tyrosine; the difference in the effectiveness of the three amino-acids is, then, understandable from their increasing distance from the point of inhibition. The parent strain is much more sensitive to the inhibitor than the mutants. This is to be expected, as the inhibition is dependent upon the ratio of metabolite to anti-metabolite. In the parent strain, the amino-acids are probably utilized about as quickly as they are formed, and do not accumulate; in the mutant, on the other hand, one has to administer the preformed amino-acid and this increases the metabolite: anti-metabolite ratio. Similar observations have been made by Volcani & Snell (1948) in the case of the canavanine inhibition of arginine-requiring micro-organisms.

In accordance with this picture, the quantity of PAPA necessary for an equal inhibition of the tyrosineless mutant is greater than that needed for the phenylalanineless mutant, the tryptophanless mutant, or the wild type.

The results agree with those of Burckhalter & Stephens (1951) and of Frieden, Stansel & Dittmer (1951), but not with the earlier claim of Elliot, Fuller & Harington (1948) that PAPA inhibits *Esch. coli* only in concentrations of 200 μg./ml.

In contrast to the aromatic amino-acids, L-arginine, L-cystine and L-glutamic acid do not relieve the inhibition of *Esch. coli* by PAPA. It is thus not possible to assume, as Beerstecher & Shive (1947*b*) have done, that these amino-acids are precursors of a metabolite which functions in the biosynthesis of phenylalanine, unless one wishes to assume that PAPA, in addition to the effect discussed here, also interferes with the conversion of these aliphatic amino-acids into phenylalanine.

p-*Nitrophenylalanine* (*PNPA*). The competitive inhibition of the tyrosineless mutant by this substance proves that the point of attack is the utilization of tyrosine. The lack of response to PNPA, shown by the wild type of *Esch. coli* and by its other mutants, must then be explained by the assumption that these strains are capable of synthesizing the metabolic product of tyrosine by a pathway which circumvents tyrosine, e.g. directly from phenylalanine.

The experiments permit the further conclusion that the wild strain, the phenylalanineless, the tryptophanless and the triply deficient mutant, at least, do not reduce PNPA to PAPA (as the latter inhibits these strains). This difference between the two substituted phenylalanines is interesting in connexion with the observation (Lampen & Peterson, 1943; Housewright & Koser, 1944) that in *Clostridium acetobutylicum*, *p*-nitrobenzoic acid replaces *p*-aminobenzoic acid as a growth factor. Experiments with PNPA also have been reported by Woolley (1950), by Burckhalter & Stephens (1951) and by Frieden *et al.* (1951).

p-*Fluorophenylalanine* (*PFPA*). This compound acts on the utilization of phenylalanine, as indicated by the fact that these two substances show a competitive antagonism in the phenylalanineless and the triply deficient mutants and that, in the wild strain, the reversal of the PFPA inhibition is most effective with phenylalanine. Similarly, recent experiments with PFPA have shown it to interfere with phenylalanine metabolism in rats (Armstrong & Lewis, 1951*a*), *Lactobacillus arabinosus* (Atkinson *et al.* 1951), *Pseudomonas aeruginosa* (Martin & Mon, 1949) and *Saccharomyces cerevisiae* (Armstrong & Lewis, 1951*b*).

Substitution of a hydrogen atom by fluorine produces an inhibitor of the reaction through which this hydrogen atom is replaced by hydroxyl. In this connection, it is recalled that *p*-fluorobenzoic acid also inhibits *Esch. coli* and that this inhibition is reversed by tyrosine (Sveinbjornsson & Vander Werf, 1951). The effect of fluorine substitution has

been studied extensively in the case of fluoroacetic acid (Chenoweth, 1949) and fluorocitric acid (Martius, 1949; Liebecq & Peters, 1949; Buffa & Peters, 1949; Buffa, Peters & Wakelin, 1951), and also in that of 4-amino-2-fluorobenzoic acid (Wyss, Rubin & Strandskov, 1943); it has been shown that these compounds are incorporated by the living cell instead of the corresponding fluorine-free substances. One may, therefore, well assume that *p*-fluorophenylalanine, too, might be incorporated by *Esch. coli* in metabolites which are normally built up from phenylalanine.

m-*Nitrotyrosine* (*MNT*). In the tyrosineless mutant, this substance affects the utilization of tyrosine; this is also evident from the inhibition of the triply deficient mutant and its reversal by tyrosine. This inhibition may also occur in the wild strain, but there it cannot be the only effect of the antagonist, since tryptophan is the most efficient of the aromatic amino-acids in reversing the inhibition. One must, therefore, conclude that the synthesis of tryptophan also is affected by MNT, an effect which can, of course, not be detected in the mutants which require preformed tryptophan, phenylalanine or tyrosine, respectively. The fact that the tryptophanless and the phenylalanineless mutants of *Esch. coli* remain unaffected by MNT, then tends to show that they can produce the metabolic product of tyrosine by a pathway which circumvents tyrosine and is not blocked by MNT.

o-*Hydroxyphenylalanine* (*OHPA*). The point of attack which the experiments with this substance have revealed (Volcani *et al.* 1951) is the utilization of phenylalanine, i.e. the synthesis of tyrosine from that amino-acid. The fact that the wild strain and the tryptophanless mutant do not respond to OHPA proves that these organisms possess a way of producing tyrosine by a route which by-passes the phenylalanine stage, e.g. directly from tryptophan. The triply deficient mutant, on the other hand, is inhibited, and addition of phenylalanine reverses the effect.

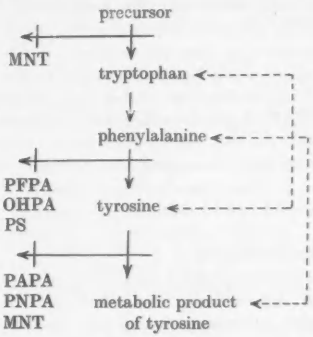
DL-*β-Phenylserine* (*PS*). Beerstecher & Shive (1946) have found that β-phenylserine inhibits *Esch. coli*, wild type, and that this inhibition is counteracted competitively by phenylalanine and one-tenth as effectively by tryptophan, whilst tyrosine was reported to be completely inactive (Beerstecher & Shive, 1947*a*). With the strain used in the present investigation (see Volcani *et al.* 1951), the position is somewhat different. The ratios of tryptophan:phenylalanine:tyrosine tend to indicate that the point of attack of β-phenylserine lies at the phenylalanine-tyrosine reaction.

Woolley (1950) has also observed that the phenylalanineless mutant (the same as used in the present investigation) is competitively inhibited by PS. His claim, that, at certain concentrations, the substance stimulated the growth of the mutant, could not be confirmed.

PS resembles *p*-fluorophenylalanine also in its inertness towards the tyrosineless mutant; the point of attack appears, in both cases, to be phenylalanine. Chemically speaking, the substitution at the β position of phenylalanine by the hydroxyl group has an effect analogous to the *p*-substitution by a fluorine atom.

Nitro- and amino-substituted β-phenylserine. These have no effect on either the wild strain of *Esch. coli* or its mutants. With regard to the *p*-nitro compound, the results disagree with those reported by Woolley (1950); this may be due to the fact that Woolley's compound posseses the *threo*-, the one used here the *erythro*-configuration (see Kopp, Larramona & Welvart, 1951; Holland & Nayler, 1952).

This discussion can be summarized in the following extension of the straight scheme, in which the crossed arrows indicate the point of attack of the inhibitor, and the dotted lines the alternative pathways of synthesis which the present investigation has suggested:



The experiments reported here cannot be fitted into any 'branched' scheme, in which the aromatic amino-acids derive independently from a common precursor.

It is noteworthy that the phenylserine derivatives studied, in spite of their inactivity towards *Esch. coli*, are not completely devoid of biochemical potentialities. Table 5 shows very strikingly for the wild type that both *m*-amino- and *m*-nitro-β-phenylserine counteract the inhibition of growth caused by PAPA, PFPA, MNT and PS. *p*-Aminophenylserine was less and *p*-nitrophenylserine not at all effective. Divatia, Gardner, Dufrenoy & Pratt (1951) reported a similar antagonistic relation between *p*-aminosalicylic acid and streptomycin in a streptomycin-resistant strain of *Esch. coli*.

As yet, any attempt to explain the effects observed in these already fairly complex systems must be speculative. Formally, either of two

mechanisms could be responsible for these effects (making the reasonable assumption that the inhibitory substances act by occupying the active spot on the surface of an enzyme involved): (*a*) the anti-antagonist withdraws the antagonist, and thus makes the active centres available for the natural substrate (one may think of formation of molecular compounds between antagonist and anti-antagonist), (*b*) the anti-antagonist lays bare masked active centres and thus creates new points of attack for the substrate.

The picture would be much clearer if it were possible to correlate the biological relationships observed with the chemical structures of the compounds concerned. An attempt at doing this seems still premature. However, the study of the sparing effects in the inhibited mutants shed some further light on the intricate situation which appears to exist.

The PFPA- and PS-inhibited phenylalanineless mutant hardly responded to tryptophan or tyrosine; on the contrary, PFPA and PS suppress even the sparing effect of tyrosine on phenylalanine, which had been observed in the uninhibited mutant. Whilst MNT did not stimulate the utilization of phenylalanine by the tyrosineless mutant, the sparing effect was quite pronounced in the PFPA-inhibited tryptophanless mutant. *p*-Aminophenylalanine (PAPA), on the other hand, stimulates the utilization, in the phenylalanineless mutant, of tryptophan and increases the sparing effect of tyrosine; in the tyrosineless mutant, it causes utilization of phenylalanine exceeding that of tyrosine. Similar effects were observed in the tryptophanless mutant.

This stimulating effect may be related to the fact, previously established, that PAPA, in certain concentrations, stimulates the growth of the tyrosineless mutant. On the other hand, the unusually high utilization of phenylalanine by the inhibited tyrosineless mutant may be due to the opening of a pathway in which phenylalanine is converted directly, not via tyrosine, into the tyrosine metabolite. The possibility of such a detour has been concluded above (p. 10) from experiments with *p*-nitrophenylalanine and *Esch. coli*, wild type, and its phenylalanineless mutant.

This hypothesis is not contradicted by the observation that tryptophan is more effective in the PAPA-inhibited phenylalanineless, than in the PAPA-inhibited tyrosineless mutant. The difference may be simply due to the fact that the way from tryptophan to phenylalanine is shorter than that to tyrosine.

The specific stimulating effect of PAPA, described above, adds a further feature to the picture of this compound, of which two properties have been reported previously: its ability to replace *p*-aminobenzoic acid completely in *Clostridium acetobutylicum* (Weizmann, 1949) and partially in the PABA-requiring mutant of *Esch. coli*, in *Lb. arabinosus*, and *Acetobacter suboxydans* (Volcani, Ben-Ishai & Bergmann, 1952), and its ability to inhibit both *Esch. coli*, wild type, and its phenylalanineless and tyrosineless mutants (see p. 10).

The results reported can be described as activation of the cellular faculties by inhibitors. The observation of Aoki, Tanemori & Noda (1951) that traces of antibiotics and drugs enhance the colony growth, the methylene-blue reducing capacity, and the virulence of certain pathogenic bacteria, represents another example of this principle.

SUMMARY

1. A synergic effect of phenylalanine and tyrosine for tryptophan has been demonstrated in the case of a tryptophanless mutant of *Escherichia coli*. For a phenylalanineless mutant, tyrosine, but not tryptophan, showed a sparing effect. For a tyrosineless mutant, neither tryptophan nor phenylalanine showed a sparing effect.

2. These results are best explained by a 'straight' scheme for the biogenetic relationships of the three aromatic amino-acids.

3. The effect of nuclear-substituted phenylalanines (*p*-amino-, *p*-nitro-, *p*-fluoro- and 4-hydroxy-3-nitrophenylalanine) as well as of *β*-phenylserine on *Escherichia coli*, wild type, and on its aromatic amino-acid-requiring mutants has been studied: *p*-aminophenylalanine, *p*-nitrophenylalanine and *m*-nitrotyrosine interfere with the utilization of tyrosine; *m*-nitrotyrosine also disturbs the synthesis of tryptophan; *p*-fluorophenylalanine and *β*-phenylserine inhibit the conversion of phenylalanine into tyrosine.

4. Alternative pathways have been suggested which lead directly (*a*) from tryptophan to tyrosine, and (*b*) from phenylalanine to the product into which the cell normally transforms tyrosine.

5. None of the strains studied is affected by *m*-nitro-, *m*-amino-, *p*-nitro- or *p*-amino-*β*-phenylserine. However, the *m*-amino and *m*-nitro compounds counteract the inhibition caused by *p*-amino- and *p*-fluoro-phenylalanine, *m*-nitrotyrosine and *β*-phenylserine in *Esch. coli*, wild type.

6. The following sparing effects have been observed in the mutants, inhibited by *p*-aminophenylalanine. (*a*) Tryptophan shows a much stronger sparing effect in the phenylalanineless mutant, when the latter is inhibited than without such inhibition. (*b*) Tyrosine shows a strong sparing effect on phenylalanine in the inhibited phenylalanine mutant. (*c*) Phenylalanine is more active than tyrosine in restoring the growth of the inhibited tyrosineless mutant of *Esch. coli*.

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The Occurrence of *n*-Decanoic Acid in Mutton Fat

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A number of fatty acid composition analyses have been made on animal body fats (see Hilditch, 1947), but the trace fatty acid constituents of these fats have not been fully investigated. The analyses reported by Hilditch (1947) show that most animal body fats, including those of sheep (Hilditch & Pedelty, 1941) contain saturated fatty acids ranging from C_{14} to C_{18} together with trace amounts of C_{12} acids. Although *n*-decanoic (capric) acid has been reported to be present in milk fats (Chevreul, 1823; Dhingra, 1933, 1934; Hilditch & Paul, 1936; Hilditch, 1947), in coconut and other palm-kernel oils (Görgey, 1848; McKinney & Jamieson, 1938), in the seed fats of a number of plants, including the elm (Schuette & Lunde, 1936), in the head oil of sperm whales (Hilditch & Lovern, 1928), and in wool grease (Weitkamp, 1945), it has not formerly been isolated from the animal body fats of animals receiving a natural diet. Evidence indicating its presence as a minor constituent of the body fats of pigs which had been fed a diet of only buttermilk, was, however, submitted by de la Mare and Shorland (1946). These authors considered that small amounts of low-molecular-weight saturated acids were assimilated from the buttermilk diet and deposited unchanged in the body tissue. Similarly, Longenecker & Hilditch (1938) reported trace amounts of both capric and lauric acids in the depot fats of rats which had been fed wholly on a diet of cows' milk, and Hilditch, Sime & Maddison (1942) identified 2·1 mol. % capric acid and 1·5 mol. % lauric acid in the fat of a lion. The C_{10} and C_{12} acids in this case were considered to have been derived from coconut cake which had been eaten by animals whose flesh had been fed to the lion.

Recent investigations on the external fatty tissues of old ewes have revealed that the fat contains, in addition to the known straight-chain acids, trace amounts of methyl branched-chain saturated acids (Hansen, Shorland & Cooke, 1952*a*, *b*). The present paper reports the further examination of the trace components of mutton fat and the isolation from it of *n*-decanoic acid.

EXPERIMENTAL

Details of the preliminary preparation of bulk material for this investigation were reported earlier (Hansen *et al.* 1952*a*, *b*). The procedure was briefly as follows. From the external fatty tissues of old overweight ewes the fat was extracted by mincing and steam-rendering. The extracted glycerides (saponification equiv. 286·9, I_2 value 46·6, unsaponifiable matter 0·56 %, and free fatty acids 0·4 %) after being converted to methyl esters (7·69 kg.) and hydrogenated were repeatedly crystallized from acetone at $-30°$ yielding 332·6 g. 'liquids' (I_2 value 7·0). Fractional distillation at about 0·2 mm. in a 22-plate Vigreux column resulted in a series of thirteen fractions and a residue (see Table 1, Hansen *et al.* 1952*a*). In this present paper, the first fraction only of this series, HL1 (wt. 17·0 g., saponification equiv. 234·9, I_2 value 1·7) has been submitted to detailed investigation. Fraction HL1 was distilled *in vacuo* (column E, Shorland, 1952), and yielded ten fractions and a residue (see Table 1).

Table 1. *Fractional distillation of methyl esters* (*HL*1)

(Wt. 15·9 g., saponification equiv. 234·9, I_2 value 1·7.)

Fraction	Wt. (g.)	Saponification equiv.	Iodine value (Wijs)
HL1L1	1·06	180·1	Nil
HL1L2	1·16	192·3	3·0
HL1L3	1·15	201·5	0·5
HL1L4	1·42	209·8	3·6
HL1L5	0·64	220·3	4·7
HL1L6	1·01	219·8	3·7
HL1L7	1·54	228·5	1·5
HL1L8	1·78	239·5	1·5
HL1L9	1·97	242·4	1·3
HL1L10	2·83	240·4	0·9
HL1LR*	0·77	258·7	24·0

* Residue excluding unsaponifiable matter. Saponification equiv. 233·6 (acid).

The methyl esters of fraction HL1L2, denoted H57, (wt. 1·16 g., saponification equiv. 192·3, I_2 value 3·0; m.p. (acids) 28·5°) were then submitted to chromatographic separation by elution with light petroleum (b.p. 50–60°) through a column packed with alumina (see Table 2).

On the basis of their having almost identical refractive indices (see Table 2) methyl ester fractions H57*b*, H57*c*, H57*d* and H57*e* were bulked and denoted H65 (wt. 0·62 g., saponification equiv. 190·8, m.p. $-11·7°$). Further purification of fraction H65 was effected by means of repeated low-temperature crystallization of its fatty acids (wt. 0·52 g., m.p. 29·0°) from light petroleum (b.p. 50–60°) and from methanol, yielding fraction H65S2LL (wt. 0·34 g., saponification equiv. 171·9, m.p. 31·2°). (See Table 3).

The chemical and physical characteristics of fraction H65S2LL are recorded in Table 4.

Two further fractions HL1L1SLS (0·21 g., m.p. 30·5°) and HL1L1LSSL (0·23 g., m.p. 30·2°) were independently derived from HL1L1 (Table 1) by low-temperature fractional crystallization.

Table 2. *Chromatographic separation of the methyl esters of fraction H57*

(Wt. 1·16 g., saponification equiv. 192·3, I_2 value 3·0.)

Fraction	Eluent (Light petroleum b.p. 50–60°) (ml.)	Wt. (g.)	Refractive index $n_D^{20°}$
H57*a*	25	0·07	1·4298
H57*b*	25	0·42	1·4254
H57*c*	25	0·12	1·4253
H57*d*	25	0·05	1·4252
H57*e*	25	0·03	1·4252
H57*f*	50	0·03	1·4282
H57*g*	50	0·01	—

The long crystal spacing reported in this paper was determined by means of a Philips Geiger X-ray spectrometer using Kα-radiation from an iron target tube with a Mn filter. Samples were melted on a glass slide and quickly cooled.

All melting points were determined in closed capillaries and are uncorrected.

DISCUSSION

Fraction H65S2LL possessed a melting point of 31·2° in close agreement with reported values for *n*-decanoic acid, namely 31·3° (Deffet, 1931), 31·19° (Meyer & Reid, 1933) and 30·9° (Weitkamp, 1945). Saturation of the fraction is indicated by the zero iodine value. Evidence of molecular weight is furnished by saponification equivalent (171·9) and combustion analyses (C, 69·9; H, 11·5%) both agreeing closely with the requirements for a saturated fatty acid $C_{10}H_{20}O_2$ (saponification equiv. 172·3; C, 69·7, H, 11·7%). The X-ray long spacing of fraction H65S2LL (22·8A.) agrees within experimental error (±0·5A.) with the value of 23·02A. recorded by Slagle & Ott (1933) for a melted specimen of *n*-decanoic acid. This figure distinguishes it from *n*-undecanoic acid (25·32A.) and from *n*-dodecanoic acid (27·18A.). When admixed with an equal quantity of authentic capric acid (m.p. 30·0°) fraction H65S2LL gave a mixed melting point of 30·0°. The chemical and physical data presented above are consistent with the fraction isolated in this work being *n*-decanoic acid.

The occurrence of capric acid in the back fat of pigs which had been fed solely on buttermilk, was attributed by de la Mare & Shorland (1946) to assimilation from the diet and direct deposition. On this hypothesis it is not surprising that as capric acid is present in the milk fat of sheep (Dhingra, 1933), it is also present in trace amounts in their body fats. (In this investigation capric acid was estimated to be present to the extent of about 0·1% of the total fatty acids.) It would be expected to occur in even greater quantities in the depot fats of lambs as well as in the young of certain other mammals, such as goats, horses, camels, buffaloes and humans (Hilditch, 1947), of whose milk fats it is a component.

The storage of dietary fatty acids below C_{16} has already been indicated, but the evidence suggests that, possibly owing to more rapid oxidation, the

Table 3. *Purification of fatty acids (H65) by means of fractional crystallization*

(Wt. 0·52 g., m.p. 29·0°, saponification equiv. of methyl esters 190·8.)

Fraction	Conditions of crystallization	Soluble fraction	Wt. (g.)	M.p. (°)	Insoluble fraction	Wt. (g.)	M.p. (°)
H65	Light petroleum, 40 vol., −70°	H65L	0·06	10·4	H65S	0·45	31·2
H65S	Light petroleum, 40 vol., −70°	H65SL	0·02	16·0	H65SS	0·42	31·8
H65SS	Methanol, 40 vol., room temp.	H65S2L	0·41	—	H65S2S	Trace	—
H65S2L	Methanol, 40 vol., −60°	H65S2LL	0·34	31·2	H65S2LS	0·03	29·0

Table 4. *Characteristics of fatty acid fraction H65S2LL*

Fraction	Wt. (g.)	M.p. (°)	Saponification equiv.	X-ray long spacing	Combustion analysis*	Iodine value (Wijs)
H65S2LL	0·34	31·2	171·9	22·8A.	C, 69·9% H, 11·5%	—
		(Value for *n*-decanoic acid 31·3)‡	(Calc. for $C_{16}H_{20}O_2$: 172·3)	(Value for *n*-decanoic acid 23·02A)†	(Calc. for $C_{10}H_{20}O_2$: C, 69·7%; H, 11·7%)	Nil

* Analysis Weiler and Strauss, Oxford.
† Determination by Slagle & Ott (1933).
‡ Determination by Deffet (1931).

efficiency of storage diminishes with decreasing molecular weight (cf. Shorland & de la Mare, 1945) so that with the development of more refined techniques, it might be feasible to demonstrate that acids of molecular weight still lower than that of capric acid can occur in mutton tallow albeit in still smaller proportions. At the present time, however, the field of investigation relating to the trace constituents of fats remains relatively unexplored.

SUMMARY

n-Decanoic acid has been isolated in trace amounts from the external fatty tissue of sheep.

We are indebted to Dr F. B. Shorland, Director of this Laboratory, for his suggestions and co-operation, and to Mr M. Fieldes, Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand, for the X-ray analysis reported in this paper.

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The Biological Action of Substances Related to Thyroxine

4. THE THYROXINE-INHIBITORY PROPERTIES OF A SERIES OF 4-HYDROXY-3:5-DIIODOBENZOATES OF GLYCOLS

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During the course of a study of the physico-chemical properties of a series of *n*-alkyl 4-hydroxy-3:5-diiodobenzoates briefly reported by Maclagan, Sheahan & Wilkinson (1951), it was found that high antithyroxine activity was paralleled by oil:water partition coefficients relatively in favour of the water phase and by relatively rapid rates of alkaline hydrolysis. This work will be discussed later, but it appeared that suitable hydroxyalkyl esters might well show a greater affinity for the aqueous phase and might therefore be expected to exhibit enhanced antithyroxine activity. A number of such substances and several polyesters of certain glycols have been prepared and tested for thyroxine-inhibitory action.

Since *p*-hydroxybenzoic acid does not form the corresponding acid chloride with such reagents as phosphorus oxychloride or thionyl chloride, the required *p*-hydroxybenzoates could not conveniently be prepared directly from this acid. However, when the phenolic hydroxyl group was protected with an acyl group, acid chlorides were readily formed on treatment with thionyl chloride. Because of the ease with which it could subsequently be removed, the carbethoxyl group proved to be the most satisfactory protection for the hydroxyl group. The crude *p*-carbethoxyoxybenzoyl chloride reacted readily with 2 equivalents of the glycol to give the appropriate hydroxyalkyl *p*-carbethoxyoxybenzoate as principal product, or with 1 equivalent to form mainly the bis-ester. In each case the main product was contaminated to some extent with the alternative product; however, separation by fractional distillation presented no difficulty.

The carbethoxyl groups were removed by treatment with sodium hydroxide and the resulting *p*-hydroxybenzoates were iodinated by the method used by Sheahan, Wilkinson & Maclagan (1951). By this means was obtained a series of five *ω*-hydroxyalkyl 4-hydroxy-3:5-diiodobenzoates (*β*-hydroxyethyl to *ζ*-hydroxyhexyl). The five corresponding polymethylene bis-esters were also prepared. In a similar manner, the monoesters were obtained from butane-2:3-diol and propane-1:2-diol.

It seemed that certain glycerol 4-hydroxy-3:5-diiodobenzoates might also be worthy of investigation. Accordingly, *iso*propylideneglycerol was treated with *p*-carbethoxyoxybenzoyl chloride, and the resulting ester hydrolysed with alkali to *iso*propylideneglycerol *p*-hydroxybenzoate. This substance reacted with iodine under the usual conditions to form the 3:5-diiodo derivative, which underwent hydrolysis with mineral acid giving the required glycerol 1-(4-hydroxy-3:5-diiodobenzoate). The same product was obtained when the *iso*propylideneglycerol *p*-hydroxybenzoate was hydrolysed with mineral acid and the resulting glycerol 1-ester iodinated.

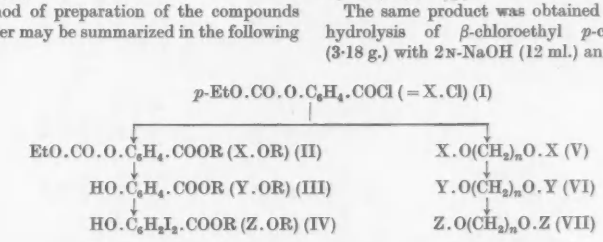
Some difficulty was experienced in separating the products of the reaction of *p*-carbethoxyoxybenzoyl chloride and glycerol. A gum which resisted distillation at 250° at 10^{-4} mm. Hg was obtained. We did not therefore pursue our plan to prepared the glycerol bis- and tris-esters.

EXPERIMENTAL

Chemical methods

Analyses were by Weiler and Strauss, Oxford. Melting points are not corrected.

The general method of preparation of the compounds described in this paper may be summarized in the following scheme:



p-Carbethoxyoxybenzoyl chloride (I). *p*-Carbethoxyoxybenzoic acid (Fischer & Freudenberg, 1910; 21 g.) and thionyl chloride (20 ml.) were heated under reflux in an oil bath at 90–100° for 1 hr. The excess thionyl chloride was removed by distillation, the last traces under reduced pressure. The residual acid chloride was not purified, but was satisfactory for use in esterification. A fresh supply was prepared for each experiment.

Preparation of hydroxyalkyl p-*carbethoxyoxybenzoates* (II). The method used may be illustrated by a description of the procedure for the *β*-hydroxyethyl ester. The acid chloride (I) (from 21 g. of the acid) was heated with ethylene dichloride (15 ml.) and ethylene glycol (9 g., 1·5 mol. equiv.) at 120° for 5 hr., mechanical stirring being employed throughout. After cooling, the mixture was treated with ice and NaOH. The product was extracted into ether, washed with water, dried (Na_2SO_4) and freed from solvent. The *β*-hydroxyethyl *p*-carbethoxyoxybenzoate (17·0 g.) distilled at 136–140°/0·05 mm. Hg, $n_D^{18°}$, 1·5173. It was not analytically pure, but gave the required *p*-hydroxybenzoate on hydrolysis.

The *β*-chloroethyl ester was prepared similarly, except that the acid chloride was soluble in the ethylene chlorohydrin (12 g., 1·5 mol. prop.), and the ethylene dichloride as solvent and the mechanical stirrer were unnecessary. The crude product was isolated in the same manner as the hydroxyethyl derivative.

β-Chloroethyl p-*carbethoxyoxybenzoate* (21·4 g., 79%) distilled at 148–149° and 0·5 mm. Hg as a colourless oil, which solidified on cooling to a colourless waxy solid, m.p. 20–21°. (Found: C, 52·8; H, 4·8; Cl, 13·4. $C_{12}H_{13}O_5Cl$ requires C, 52·9; H, 4·8; Cl, 13·0%.)

The residues remaining after distillation of the *p*-carbethoxyoxybenzoates of the higher glycols were found to consist of the corresponding bis-esters, which were induced to crystallize by treatment with ethanol.

Preparation of hydroxyalkyl p-*hydroxybenzoates* (III). The general method for the hydrolysis of the *p*-carbethoxyoxybenzoates is exemplified by that used for the *β*-hydroxyethyl ester. The *p*-carbethoxyoxybenzoate (16·5 g.) was dissolved in ethanol (65 ml.) and treated with 2N-NaOH (65 ml.) on a steam bath for 30 min. The ethanol was removed by distillation and the cooled aqueous residue extracted with ether to remove alkali-insoluble matter. The aqueous alkaline layer was then treated with either CO_2 or acetic acid to adjust the pH to 7. The solid which separated was appreciably soluble in water and was extracted into ether. On removal of the solvent, *β-hydroxyethyl* p-*hydroxybenzoate* (6·56 g., 56%) was obtained. This crystallized from benzene containing a little methanol in colourless prisms, m.p. 140–141°. (Found: C, 59·5; H, 5·3. $C_9H_{10}O_4$ requires C, 59·3; H, 5·5%.)

The same product was obtained in better yield by the hydrolysis of *β*-chloroethyl *p*-carbethoxyoxybenzoate (3·18 g.) with 2N-NaOH (12 ml.) and ethanol (15 ml.) on a steam bath for 30 min. The product (1·98 g., 85%), isolated in the same manner, was identical with that described above. (Found: C, 59·3; H, 5·6%.)

The *dibenzoyl* derivative crystallized from ethanol in colourless needles, m.p. 127°. (Found: C, 70·5; H, 4·5. $C_{22}H_{18}O_6$ requires C, 70·8; H, 4·6%.)

Iodination of p-*hydroxybenzoates* (IV). *Preparation of β-hydroxyethyl* 4-*hydroxy*-3:5-*diiodobenzoate.* The *p*-hydroxybenzoate (2·5 g.) and iodine (3·47 g.) were dissolved in ethanol (25 ml.) and heated under reflux with a solution of KIO_3 (0·975 g.) in water (10 ml.) for 1·5 hr. The solution was

Table 1. *Properties of other hydroxyalkyl esters and related substances*

(Type II, EtO.CO.O.C_6H_4.COOR; type III, HO.C_6H_4.COOR; type IV, HO.$C_6H_2I_2$.COOR.)

Compound		Yield	Properties		Mol. formula	Analyses					
						Found			Required		
Type	R	(%)	B.p./mm. Hg	n_D		C	H (%)	I	C	H (%)	I
II	CH_3.CHOH.Me	67	134–137°/0·5	—	$C_{12}H_{16}O_6$	—	—	—	—	—	—
II	CH_3.CH_2.CH_2OH	65	152–154°/0·04	$1·5101^{19°}$	$C_{13}H_{16}O_6$	57·7	6·2	—	58·3	6·0	—
II	CH_3.$[CH_2]_2$.CH_2OH	39*	168–171°/0·05	$1·5060^{17°}$	$C_{14}H_{18}O_6$	59·8	6·4	—	59·6	6·4	—
II	CHMe.CHOH.Me	69	144–146°/0·1	$1·5052^{19°}$	$C_{14}H_{18}O_6$	58·4	6·1	—	59·6	6·4	—
II	CH_3.$[CH_2]_3$.CH_2OH	34*	196–198°/0·5	$1·5066^{19°}$	$C_{15}H_{20}O_6$	61·4	7·1	—	60·8	6·8	—
II	CH_3.$[CH_2]_4$.CH_2OH	50*	177–180°/0·05	$1·5047^{17°}$	$C_{16}H_{22}O_6$	62·1	7·0	—	61·9	7·1	—
II	CH_3.CH(O—CH_2)OCMe_2†	60	157°/0·4	$1·5003^{22·5°}$ (*a*)	$C_{16}H_{20}O_7$	58·8	6·1	—	59·2	6·2	—
			M.p.	Cryst. form.							
III	CH_3.CHOH.Me	76	86–87°	Needles from benzene-ethanol (*b*)	$C_{10}H_{12}O_4$	61·3	6·0	—	61·3	6·1	—
III	CH_3.$[CH_2]_2$.CH_2OH	51	91–93°	Irreg. from benzene (*c*)	$C_{10}H_{12}O_4$	61·0	6·25	—	61·3	6·1	—
III	$CH_3[CH_2]_2$.CH_2OH	59	98°	Squat prisms from CH_2Cl_2.EtOH	$C_{11}H_{14}O_4$	63·0	6·7	—	62·9	6·7	—
III	CHMe.CHOH.Me	84	107°	Prisms from $CHCl_3$	$C_{11}H_{14}O_4$	62·9	6·7	—	62·9	6·7	—
III	CH_3.$[CH_2]_3$.CH_2OH	66	115–116°	Iridescent plates from $CHCl_3$ (Soxhlet)	$C_{12}H_{16}O_4$	64·2	7·1	—	64·3	7·15	—
III	CH_3.$[CH_2]_4$.CH_2OH	80	102–103°	Prisms from $CHCl_3$	$C_{13}H_{18}O_4$	65·5	7·5	—	65·5	7·6	—
III	CH_3.CH(O—CH_2)OCMe_2†	68	123–124°	Prisms from benzene	$C_{13}H_{16}O_5$	61·8	6·0	—	61·9	6·3	—
IV	CH_3.CHOH.Me	80	160–161°	Needles from 90% aqueous EtOH	$C_{10}H_{10}O_4I_2$	27·1	2·2	56·8	26·8	2·2	56·7
IV	CH_3.CH_2.CH_2OH	72	154–155°	Needles from 80% aqueous EtOH	$C_{10}H_{10}O_4I_2$	27·1	1·9	56·85	26·8	2·2	56·7
IV	CH_3.$[CH_2]_2$.CH_2OH	75	144–145°	Rhombs from 80% aqueous EtOH	$C_{11}H_{12}O_4I_2$	28·9	2·5	55·0	28·6	2·6	55·0
IV	CHMe.CHOH.Me	73	176°	Prisms from 80% aqueous EtOH	$C_{11}H_{12}O_4I_2$	29·0	2·9	55·0	28·6	2·6	55·0
IV	CH_3.$[CH_2]_3$.CH_2OH	76	131°	Plates from 80% aqueous EtOH	$C_{12}H_{14}O_4I_2$	30·9	2·9	53·6	30·3	2·95	53·4
IV	CH_3.$[CH_2]_4$.CH_2OH	78	85–86°	Cubes from benzene	$C_{13}H_{16}O_4I_2$	32·3	3·2	51·7	31·9	3·3	51·85
IV	CH_3.CH(O—CH_2)OCMe_2†	76	93°	Plates from 80% aqueous EtOH	$C_{13}H_{14}O_5I_2$	31·3	2·95	50·1	30·95	2·8	50·4
IV	CH_2.CHOH.CH_2OH†	69	128–129°	Plates from 80% aqueous MeOH	$C_{10}H_{10}O_5I_2$	25·7	2·3	54·8	25·8	2·15	54·7

(*a*) M.p., 36–37°, white waxy solid. (*b*) B.p. 162–165°/0·5 mm. (*c*) B.p. 171–172°/0·04 mm.

* In addition to the reported yields of hydroxyalkyl *p*-carbethoxyoxybenzoates, the following yields of polymethylene bis-esters were produced simultaneously in these experiments: tetramethylene, 14; pentamethylene, 31; and hexamethylene, 20%.

† See text (p. 20) for discussion of alternative routes to the glycerol 1-ester.

Table 2. *Properties of other polymethylene esters*

(Type V $(EtO.CO.O.C_6H_4.COO)_2>(CH_2)_n$; type VI $(HO.C_6H_4.COO)_2>(CH_2)_n$; type VII $(HO.C_6H_2I_2.COO)_2>(CH_2)_n$.)

Compound		Yield	Properties			Analyses					
						Found			Required		
Type	n	(%)	M.p.	Cryst. form	Mol. formula	C	H (%)	I	C	H (%)	I
V	3	72	64°	Plates from EtOH	$C_{23}H_{24}O_{10}$	60·1	5·2	—	60·1	5·2	—
V	4	60	68°	Needles from EtOH	$C_{24}H_{26}O_{10}$	61·2	5·2	—	60·7	5·5	—
V	5	84	58–60°	Needles from EtOH	$C_{25}H_{28}O_{10}$	61·5	5·7	—	61·5	5·75	—
V	6	79	73–74°	Irreg. from EtOH	$C_{26}H_{30}O_{10}$	62·4	5·7	—	62·2	6·0	—
VI	2	83	245–246°	Plates from MeOH	$C_{16}H_{14}O_6$	63·4	4·75	—	63·3	4·6	—
VI	3	88	200–201°	Needles from MeOH	$C_{17}H_{16}O_6$	64·7	5·1	—	64·6	5·1	—
VI	4	89	194–195°	Iridescent plates from 90% aqueous EtOH	$C_{18}H_{18}O_6$	65·2	5·5	—	65·4	5·5	—
VI	5	88	(*a*) 96–100° (decomp.)	Plates from 90% aqueous MeOH	$C_{19}H_{20}O_6.2CH_3OH$	60·8	6·55	—	61·8	6·85	—
			(*b*) 132–133°	Amorphous, from (*a*) by heating at 70°	$C_{19}H_{20}O_6$	66·2	5·6	—	66·3	5·8	—
VI	6	98	183–184°	Iridescent plates from 80% aqueous EtOH	$C_{20}H_{22}O_6$	67·0	6·05	—	67·0	6·15	—
VII	3	87	227–228° (decomp.)	Iridescent plates from dioxan-EtOH	$C_{17}H_{12}O_6I_4$	25·8	1·55	62·5	24·9	1·5	61·9
VII	4	81	245–246° (decomp.)	Needles from dioxan-EtOH	$C_{18}H_{14}O_6I_4$	25·9	2·1	60·7	25·9	1·7	60·9
VII	5	77	(*a*) 130° (decomp.)	Plates from EtOH	$C_{19}H_{16}O_6I_4.C_2H_5OH$	27·9	1·9	57·6*	28·2	2·46	56·8
			(*b*) 162°	Amorphous, from (*a*) by heating at 100°	$C_{19}H_{16}O_6I_4$	26·7	1·9	60·0	26·9	1·9	59·9
VII	6	89	187°	Prisms from *n*-butanol	$C_{20}H_{18}O_6I_4$	28·2	2·0	59·1	27·9	2·1	58·9

* Loss in wt. at 100°/0·05 mm. Hg, 3·8%. Required for loss of C_2H_5OH, 5·1%.

decolorized with $Na_2S_2O_3$ and diluted with 1 vol. of water. The *ester* (4·47 g., 75%), which separated on cooling, was collected, washed with water until free from iodides and dried at 90°. After crystallization from ethanol, it formed colourless needles, m.p. 164–165°. (Found: C, 25·3; H, 2·0; I, 59·0. $C_9H_6O_4I_2$ requires C, 24·9; H, 1·9; I, 58·5%.)

The *diacetyl* derivative crystallized from ethanol in colourless highly refractive needles, m.p. 120°. (Found: C, 30·2; H, 2·2; I, 49·1. $C_{13}H_{12}O_6I_2$ requires C, 30·1; H, 2·3; I, 49·1%.)

The properties of other hydroxyalkyl 4-hydroxy-3:5-diiodobenzoates prepared by the general methods outlined above are summarized in Table 1.

The esters of *iso*propylideneglycerol are also included, but as alternative methods of converting these to the glycerol 1-ester were investigated, the results obtained are briefly reported below.

Glycerol 1-p-*hydroxybenzoate*. *iso*Propylideneglycerol *p*-hydroxybenzoate (Table 1) (3·88 g.) was treated with gently boiling N-HCl (30 ml.) for 30 min., the liberated acetone being allowed to escape. After concentration to approx. 10 ml., the solution was refrigerated overnight. The crystalline *product* (2·65 g., 90%) was collected and dried at 100°. Recrystallization from water (or methanol) gave fine colourless needles, m.p. 157°. (Found: C, 56·6; H, 5·6. $C_{10}H_{12}O_5$ requires C, 56·6; H, 5·7%.)

Glycerol 1-4′-*hydroxy*-3′:5′-*diiodobenzoate*. *iso*Propylideneglycerol 4-hydroxy-3:5-diiodobenzoate (112 mg.) was moistened with N-HCl (2 ml.) and heated to 100°. The solid melted, acetone was liberated and the oil solidified again. After cooling the solid was filtered off and washed with water. The product (98 mg., 95%), crystallized from 80% (v/v) aqueous methanol, melted at 128–129° and proved to be identical with that obtained by iodination of the *p*-hydroxybenzoate (Table 1).

*Preparation of polymethylene bis-*p-*carbethoxyoxybenzoates* (V). *Dimethylene bis-ester*. The acid chloride (I) from *p*-carbethoxyoxybenzoic acid (21 g.) was dissolved in ethylene dichloride (20 ml.) and treated with ethylene glycol, previously dried by heating at 150° for 30 min. (3·25 g.). The mixture was heated in an oil bath at 120° for 4 hr., mechanical stirring being employed throughout. The cooled mixture was treated with ice and NaOH, and the product extracted into benzene. After drying, the solvents were removed and the residue freed from the hydroxyethyl ester by heating at 180° at 0·1 mm. Hg. The residue crystallized on treatment with ethanol, and, after recrystallization from the same solvent, the required *ester* (11·0 g.) was obtained in colourless needles, m.p. 102°. (Found: C, 59·3; H, 4·75. $C_{22}H_{22}O_{10}$ requires C, 59·3; H, 4·95%.)

*Polymethylene bis-*p-*hydroxybenzoates* (VI). The method was essentially that used for hydrolysing the hydroxyalkyl *p*-carbethoxyoxybenzoates, but owing to the sparing solubility of the sodium derivatives of the products, it was not practicable to extract alkali-insoluble material with ether. The pH of the reaction mixture had to be adjusted to 7 while still hot.

*Iodination of the bis-*p-*hydroxybenzoates* (VII). *Dimethylene bis-*4-*hydroxy*-3:5-*diiodobenzoate*. The bis-*p*-hydroxybenzoate (0·604 g.) and iodine (1·016 g.) were dissolved in hot ethanol (25 ml.) and treated with KIO_2 (0·286 g.) in water (10 ml.). After heating under reflux for 1·5 hr., the mixture was decolorized with $Na_2S_2O_3$ and diluted with water (50 ml.). The solid (1·36 g., 84%) was collected, washed with water and dried at 100°. It crystallized from dioxan-ethanol in colourless needles, m.p. 244° (decomp.). (Found: C, 23·8; H, 1·3; I, 63·6. $C_{16}H_{10}O_6I_4$ requires C, 23·9; H, 1·25; I, 64·1%.)

The other polymethylene bis-4-hydroxy-3:5-diiodobenzoates prepared by similar methods are described in Table 2.

Biological methods

The hydroxydiiodobenzoates were tested for antithyroxine activity in mice by the O_2 consumption method of Maclagan & Sheahan (1950) including the improvements suggested by Sheahan *et al.* (1951). The results are presented mainly in terms of the minimum effective dose (M.E.D.), which is the lowest dose found to produce a statistically significant inhibition ($P < 0·02$) of the O_2 consumption in thyroxine-treated mice.

RESULTS

Thyroxine-inhibitory action was displayed in varying degrees by most of the hydroxyalkyl esters tested. In general, increasing chain length of the alkyl group was paralleled by a substantial diminution in activity (Table 3). The hydroxyethyl and the two hydroxypropyl esters caused significant

Table 3. *Antithyroxine activities of some hydroxyalkyl esters of* 4-*hydroxy*-3:5-*diiodobenzoic acid*

(Two groups of eight mice each were used for controls, thyroxine-treatment and thyroxine + inhibitor treatment (cf. Sheahan *et al.* 1951).)

Ester	Total M.E.D. (mg./kg.) in mice	Mean percentage inhibition of thyroxine effect at M.E.D.	Maximum dose (mg./kg.) which failed to produce a response
β-Hydroxyethyl	25	23	12
β-Hydroxy-*n*-propyl	25	20	12
γ-Hydroxy-*n*-propyl	25	26	12
1-Glycerol	—	—	400
δ-Hydroxy-*n*-butyl*	25	35	12
β-Hydroxy-α-methylpropyl	400	86	200
ε-Hydroxy-*n*-amyl	400	35	200
ζ-Hydroxy-*n*-hexyl	400	24	200

Inhibition was significant ($P < 0·01$) for all compounds except the glycerol ester.

* The results obtained with this compound were somewhat variable. Some batches of mice gave the figures quoted whilst with other batches no antithyroxine effect was observed even at higher dose levels.

reductions in the thyroxine responses at total doses of 25 mg./kg. and were thus as effective as the *n*-butyl ester, the most potent compound hitherto observed during the course of this study. The 3-hydroxy-*sec.*-butyl ester and those derived from the higher glycols showed activity only in high dosage.

Table 4. *Effect of the introduction of a hydroxyl group on the antithyroxine activity of some alkyl 4-hydroxy-3:5-diiodobenzoates*

Ester	Total minimum effective doses in mice (mg./kg.): Hydroxyalkyl ester (*a*)	Alkyl ester (*b*)	Ratio of potencies (*b*/*a*)
Ethyl	25 (β)	50*	2
n-Propyl	25 (β)	50*	2
	25 (γ)	—	2
	>400 ($\beta\gamma$-dihydroxy)	—	<0·125
n-Butyl	25† (δ)	25*	1
sec.-Butyl	400 (β)	100‡	0·25
n-Amyl	400 (ϵ)	400*	1
n-Hexyl	400 (ζ)	400*	1

* Sheahan *et al.* (1951).
† See footnote to Table 3.
‡ Wilkinson, Sheahan & Maclagan (1951).

Table 5. *The antithyroxine activities of some polymethylene bis-4-hydroxy-3:5-diiodobenzoates*

Ester	Total M.E.D. (mg./kg.) in mice	Percentage inhibition of thyroxine effect at M.E.D.	Maximum dose (mg./kg.) which failed to produce a response
Dimethylene	25	35*	12
Trimethylene	—	—	400
Tetramethylene	400	29†	200
Pentamethylene	200	41*	100
Hexamethylene	—	—	800

* Significance $P<0·01$. † Significance $P<0·02$.

The effect of the substitution of a hydroxyl group on the thyroxine-inhibitory activity of a number of alkyl 4-hydroxy-3:5-diiodobenzoates is shown in Table 4 to be relatively slight. The slight enhancement observed in the case of the hydroxyethyl and hydroxypropyl esters was not found in the higher esters, and the β-hydroxyl group substituted in the *sec.*-butyl ester actually produced a fourfold diminution in activity. A second hydroxyl group in the alkyl chain completely abolished the activity of the propyl ester, for the glycerol ester displayed no action at a dosage of 400 mg./kg.

Table 5 shows that there is no correlation between the length of the polymethylene chain and the antithyroxine activity of the polymethylene bis-4-hydroxy-3:5-diiodobenzoates. The dimethylene compound was highly active whilst the tetramethylene and pentamethylene esters were feebly to moderately active; the remaining members showed no effect whatever.

SUMMARY

1. A series of hydroxyalkyl 4-hydroxy-3:5-diiodobenzoates and the corresponding polymethylene bis-esters have been prepared and their antithyroxine activities determined by the oxygen consumption method in mice.

2. The introduction of the hydroxyl group into the alkyl group caused a slight enhancement of the activity of the lower esters but little change in that of the longer-chain molecules.

3. The presence of two hydroxyl groups in the alkyl chain abolished the antithyroxine effect, for the glycerol ester was inactive at high dosage.

4. The lower hydroxyalkyl esters and the dimethylene bis compound were active at a total dose of 25 mg./kg. and thus equalled the potency of the *n*-butyl ester, the most effective compound hitherto obtained.

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Studies on Porphobilinogen

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The term porphobilinogen will be used in the present paper for the colourless chromogen excreted in acute porphyria, often in association with free porphyrins, their metal complexes and other pigments, and responsible for the reaction with *p*-dimethylaminobenzaldehyde (Ehrlich's aldehyde reagent) giving a chloroform-insoluble red pigment. The chromogen itself has not yet been isolated, but crude solutions have been prepared by chromatography and shown to form in acid solution a mixture of porphyrins and porphobilin, a highly polar bile pigment with properties resembling urobilin (Waldenström & Vahlquist, 1939*a*; Prunty, 1945). Waldenström & Vahlquist, on the basis of molecular weight determinations by the diffusion method, proposed that porphobilinogen was a dipyrryl compound, two molecules of which could condense together to give a bile pigment or, in accordance with the theory of Dobriner, Strain & Localio (1937), a porphyrin. Neuberger, Muir & Gray (1950) have emphasized that such a mechanism was unlikely to give rise to naturally occurring porphyrins which are of types I and III only.

Recently, Hawkinson & Watson (1952) and also Herbert (1952) have observed that ethyl acetate extracts of porphyria urines contained a colourless porphyrin precursor not reacting with *p*-dimethylaminobenzaldehyde but forming a porphyrin on irradiation with ultraviolet light or on oxidation. The work of Herbert may be interpreted as suggesting that the increased amounts of porphyrin obtained on heating crude acidified porphyria urines were due to the formation of porphyrin from this precursor as well as from porphobilinogen. However, Lowry, Schmid, Hawkinson, Schwartz & Watson (1950) claim to have prepared solutions of porphobilinogen which, on heating in acid, formed only porphobilin, and their work implies that porphobilinogen is a precursor of porphobilin only, and that solutions of porphobilinogen which give rise to porphyrin must contain a porphyrin precursor as an impurity.

It appeared of interest to determine the effect of heating on the absorption spectrum of porphobilinogen solutions under various conditions, for Grieg, Askevold & Sveinsson (1950) have shown that maximum yields of porphyrin from porphyria urines are obtained only if the urines are heated at 100° at pH 5·2, while Herbert (1952) has shown that porphyrin formation from porphobilinogen solutions is greatest in 0·05–0·2 N-hydrochloric acid. We have found that solutions of porphobilinogen prepared chromatographically give rise to at least three pigments, a porphyrin, a yellow pigment and a violet pigment, the yield of each depending on the conditions. In four urines examined we have found no evidence of the presence of a porphyrin precursor other than porphobilinogen.

Preliminary reports of this work have already been communicated to the Biochemical Society (Gray, 1951; Brockman & Gray, 1951, 1952).

MATERIALS AND METHODS

Porphobilinogen solution. Porphyria urines containing 5–10 units of porphobilinogen/ml. were chromatographed five times through alumina by the method of Prunty (1945). The units are those of Prunty (1945). Each eluate was brought to pH 6·8 with 20% (w/v) NaOH, the white precipitate was removed by centrifugation, and the solution was stored in the refrigerator until it could be rechromatographed. The final neutralized eluate contained 10–15 units of porphobilinogen/ml., i.e. it was very little more concentrated than the original urine but contained less than 0·01 mg. of urea/ml. This neutralized eluate was used throughout the work except where stated otherwise. In some later experiments the final porphobilinogen eluate in 1% acetic acid was freeze-dried to give a stable, white powder containing 4 units of porphobilinogen/mg., approximately 1% of uric acid, a trace of amino-acid and much sodium acetate which presumably arose as a result of neutralizing the eluates with NaOH.

Uricase. This was kindly supplied as a suspension in glycine buffer, pH 9·4, by Dr E. Praetorius of Copenhagen. The concentration of the enzyme in the suspension was unknown.

Glycine buffer. This buffer, pH 9·4, 0·66 M, was prepared as described by Praetorius (1949).

Acetate buffers. These were prepared according to Cole (1933).

Veronal-acetate buffer. pH 9·3. This was the modification by Neuberger & Scott (1952) of that described by Michaelis (1931).

Spectral absorption. This was measured in a Hilger Uvispek photoelectric spectrophotometer.

pH *Measurements.* These were made with a Cambridge pH meter using a glass electrode.

Porphobilinogen concentration. This was measured in units as described by Prunty (1945) using the *p*-dimethylaminobenzaldehyde reagent of Waldenström & Vahlquist (1939*b*).

EXPERIMENTAL AND RESULTS

Absorption spectrum of porphobilinogen solutions free from uric acid

Before the removal of uric acid the colourless porphobilinogen solution showed only a strong absorption maximum in the ultraviolet at 292 mμ. (Fig. 1). The result of uricase

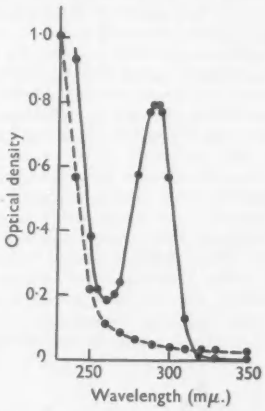

Fig. 1. Absorption spectrum of porphobilinogen solution in 0·066 M-glycine buffer, pH 9·4. ——, before destruction of uric acid; – – –, after destruction of uric acid by the addition of 0·01 ml. uricase suspension/3 ml. of porphobilinogen solution. Suitable blanks of 0·066 M-glycine buffer, pH 9·4, without and with added uricase were used.

treatment was to abolish all absorption at wavelengths longer than 250 mμ. The absorption in the region of 290 mμ., which previously was regarded as due to porphobilinogen, was obviously due to uric acid.

Changes in absorption spectrum on heating porphobilinogen solutions at pH 5·2

After heating for 1–2 min. the porphobilinogen solution became yellow and showed two absorption maxima, a strong one at 477–480 mμ. and a much weaker one in the region of 350–370 mμ. (Fig. 2*a*). On heating for a further 3 min. the absorption at these wavelengths increased and a new maximum appeared at 407 mμ. At this stage the solution fluoresced red in ultraviolet light. On further heating the solution became pink and the two absorption bands at 350–370 and 477–480 mμ. diminished in intensity. At the same time the 407 mμ. band increased in intensity and reached its maximum after about 40 min. heating (Fig. 2*b*). After 70 min. heating (curve not shown in Fig. 2*b*) the absorption at 480 mμ. was diminished further in intensity; that at 407 mμ. was also diminished. There was no change in the absorption due to uric acid which at pH 5·2 is at 286–288 mμ.

The absorption maximum at 407 mμ. was undoubtedly the Soret band of a porphyrin, for the intensity of this band increased in proportion to the red fluorescence in ultraviolet light. The position of this absorption maximum and the ether-insolubility of the porphyrin is not incompatible with its being uroporphyrin.

Effect of molarity. When freeze-dried porphobilinogen was dissolved in acetate buffers, pH 5·2, of different molarities and heated, red precipitates were formed. Fig. 3*a* and *b* show the absorption spectra of the supernatant solutions, and of the solutions of the precipitates in 0·5 N-HCl. In 0·1 M buffer the supernatant showed no significant absorption at wavelengths above 350 mμ., but the acid solutions of the precipitates showed absorption maxima at 407 and 480–500 mμ. In M buffer the supernatant showed an absorption maximum at 480 mμ. only, and the acid solutions of the precipitates showed maxima at 407 and 480 mμ.

The differences in spectra of supernatant and of acid solutions of the precipitate cannot be due to differences in pH of the solutions. None of the supernatants contained

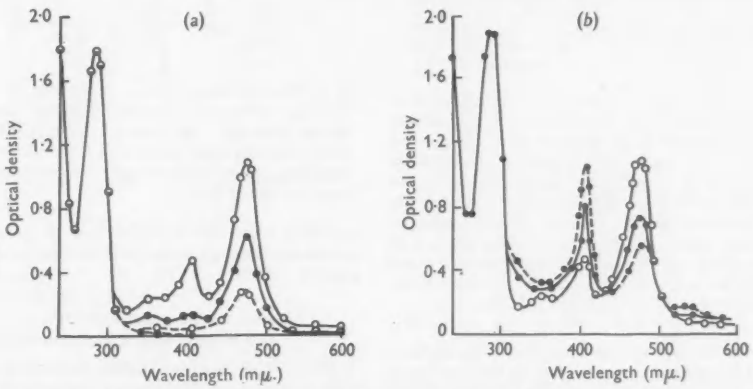

Fig. 2. Changes in absorption spectrum on heating porphobilinogen solutions at pH 5·2. Porphobilinogen solution, stored at pH 6·8 at 0° was diluted with an equal volume of 2 M-acetate buffer, pH 5·2. Samples were heated in a boiling-water bath for the times specified, and immediately cooled. (*a*) ●– – –●, before heating; ○– – –○, after heating for 1 min.; ●—●, after heating for 2 min.; ○—○, after heating for 5 min. (*b*) ○—○, after heating for 5 min.; ●—●, after heating for 20 min.; ●– – –●, after heating for 40 min.

any porphyrin as judged by the absence of absorption at 407 mμ. The pH's of supernatants and acid solutions were 5·2 and 1·0 respectively, both well on the acid side of the apparent dissociation constant of the yellow pigment (see below). The difference between the effects of heating in the two buffers may therefore be shown by plotting a composite curve obtained by summation of the optical density of each supernatant with that of the corresponding precipitate dissolved in 0·5 N-HCl (Fig. 3*c*).

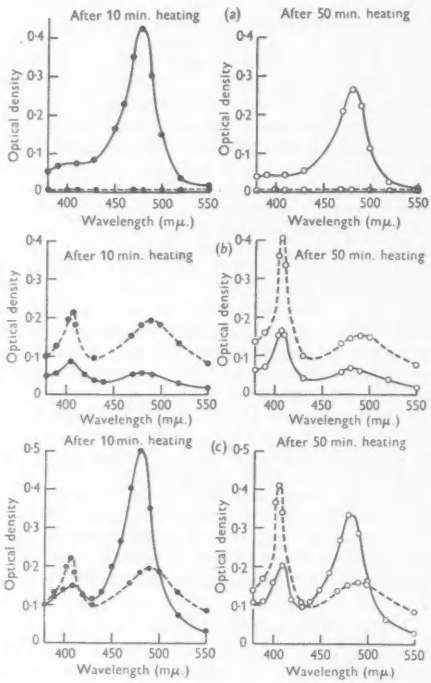

Fig. 3. Changes in absorption spectra on heating porphobilinogen solutions in 0·1 M- and M-acetate buffer, pH 5·2. (*a*) Spectral absorption of supernatants; (*b*) spectral absorption of acid solutions of precipitates; (*c*) composite curves obtained by summing the optical densities in the supernatants given in (*a*) and the acid solutions of the precipitates given in (*b*). ●– – –●, after 10 min. heating in 0·1 M-acetate buffer; ●—●, after 10 min. heating in M-acetate buffer; ○– – –○, after 50 min. heating in 0·1 M-acetate buffer; ○—○, after 50 min. heating in M-acetate buffer.

In both M and 0·1 M buffers the absorption in the region of 480 mμ. was at its maximum after 10 min. heating, but in the stronger buffer it was twice as intense as that in the weaker buffer. The maximum yield of porphyrin was obtained in both solutions after 50 min. heating, but the yield in the 0·1 M buffer was twice as great as that obtained in the M buffer. A slightly greater proportion of porphyrin was formed when the freeze-dried porphobilinogen was heated in water, the salts present sufficing to give a pH of 5·2. With solutions of molarities between 0·1 M and M the yields of the two pigments were intermediate between those obtained in the 0·1 M and M buffers. Increasing concentrations of acetate thus appeared to decrease the porphyrin yield with a proportionate increase in the yield of the yellow pigment.

When freeze-dried porphobilinogen was dissolved in M- and 0·1 M-formate and propionate buffers, pH 5·2, and heated, the results were similar to those obtained in the corresponding M- and 0·1 M-acetate buffers.

Rate of disappearance of Ehrlich reaction. Fig. 4 shows the intensity of the Ehrlich reaction after heating (for different times) the freeze-dried porphobilinogen dissolved in water, and in M-acetate buffer, pH 5·2, i.e., conditions under which porphyrin and yellow pigment production predominate, respectively. Fig. 5*a* and *b* show the percentage disappearance of porphobilinogen plotted against the corresponding intensities of the spectral absorption at 407 and 480 mμ. Porphobilinogen disappearance was apparently directly proportional to porphyrin formation in the acetate buffer, but low optical densities resulted in a considerable experimental error. Porphobilinogen disappearance was clearly not directly proportional to porphyrin formation in water, or to yellow pigment formation in either water or buffer. Analysis of the two curves reveals that the reaction

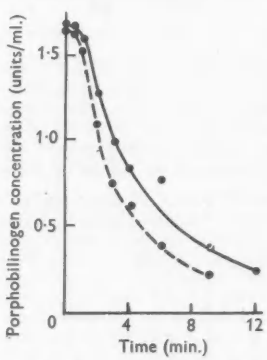

Fig. 4. Rate of disappearance of the Ehrlich reaction on heating freeze-dried porphobilinogen dissolved in: ●—●, water; ●– – –●, M-acetate buffer, pH 5·2. Samples were removed after convenient intervals for the determination of porphobilinogen concentration and of spectral absorption.

concerned with the disappearance of porphobilinogen whether in water or in buffer is neither first order nor second order.

Changes in absorption spectrum on heating porphobilinogen solutions in HCl

The series of changes occurring on heating in acid were similar to those represented in Fig. 2*a* and *b* for solutions at pH 5·2, except that maximum porphyrin formation, as indicated by the height of the absorption band at 407 mμ., occurred after only 10 min. heating. The yield of porphyrin was greatest in 0·05–0·2 N-HCl and was twice as great as the maximum yield obtained in water at pH 5·2. These obser-

vations agree with the recent findings of Herbert (1952). In acid more concentrated than 0·2 N the porphyrin appeared to be rapidly destroyed on heating. The yield of yellow pigment as indicated by the height of the 477–480 mμ. band was greatest in 2 N-HCl, and was approximately equal to the maximum yield obtained in M-acetate buffer. While this

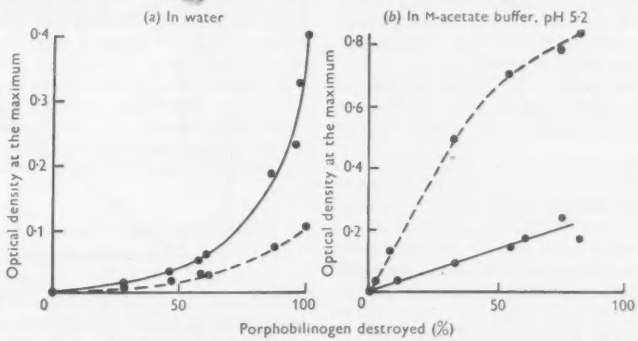

Fig. 5. Disappearance of porphobilinogen with formation of porphyrin and yellow pigment. The intensity of the Ehrlich reaction is expressed as the percentage of porphobilinogen destroyed. The porphyrin and yellow pigment are expressed in terms of their optical densities at 407 and 480 mμ., respectively. ●—●, porphyrin; ●- - -●, yellow pigment.

would suggest that the porphyrin decomposes under these conditions to form the yellow pigment, this appears unlikely in view of the more complete investigation carried out at pH 5·2.

of a spectrophotometrically detectable dissociation. The alkaline eluate was diluted with an equal volume of veronal-acetate buffer, pH 9·3, and the changes in spectral absorption were determined when the pH of the solution was varied by the addition of either 0·1 N-HCl or 0·1 N-NaOH to samples of the buffered solution. In each case the solution was made to constant volume with distilled water. No attempt was made to maintain constant ionic strength.

In solutions of pH less than 4, the yellow pigment had a strong absorption maximum at 477–478 mμ., but as the pH was increased the maximum gradually shifted to 483–484 mμ. and the optical density decreased. In very alkaline solutions a second small peak appeared at 495–497 mμ. (Fig. 6). The spectrophotometric titration curve (Fig. 7) shows that the apparent pK of the yellow pigment is 9·0.

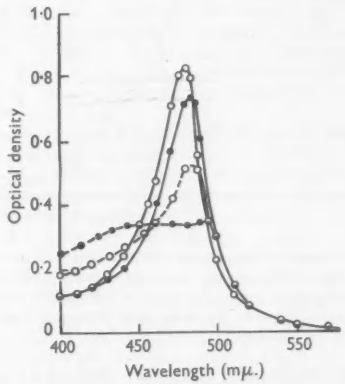

Fig. 6. Effect of pH on the absorption spectrum of the yellow pigment. ○—○, pH 2·2; ●—●, pH 7·2; ○- - -○, pH 9·3; ●- - -●, pH 10·2.

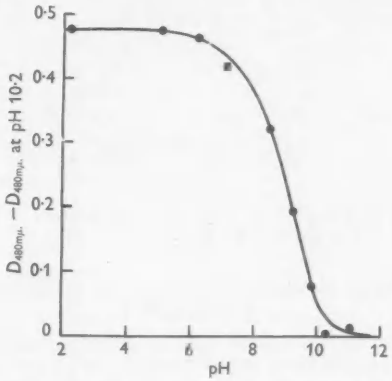

Fig. 7. Spectrophotometric titration curve of the yellow pigment. $D_{480\,m\mu.}$ = optical density at 480 mμ. at observed pH; $D_{480\,m\mu.}$ at pH 10·2 = optical density at 480 mμ., when the pH was 10·2. This was the lowest optical density recorded.

Properties of the yellow pigment

The yellow solutions obtained after heating various porphobilinogen solutions at pH 5·2 for 5–10 min. were united and passed through an alumina column. The yellow pigment which stayed at the top of the column was eluted with 0·35% (w/v) NH_3.

The eluate was greenish yellow in alkaline solution but changed to a pinkish yellow in acid, suggesting the existence

The ability of the yellow pigment to give rise to porphyrin on heating has been studied. The alkaline eluate was brought to pH 5·2 and samples were heated for 10 and 30 min. respectively. A yellow precipitate was formed, but the absorption curves of the precipitate dissolved in 0·5 N-HCl and of the corresponding supernatant showed a maximum only at 480 mμ. The absence of red fluorescence and of an absorption maximum in the region 407 mμ. showed that no porphyrin was formed. The yellow precipitate was found to be insoluble in all organic solvents. After treating with methanolic HCl it was possible to extract most of the pigment with $CHCl_3$. Attempts to crystallize the $CHCl_3$-soluble pigment have not so far been successful.

The yellow solution gave an intense green fluorescence when added to ethanolic zinc acetate, a reaction characteristic of a dipyrrylmethene and certain tetrapyrrolic compounds. It failed to couple with diazotized sulphanilic acid showing that all carbon atoms of any pyrrole rings present must bear substituents. The colourless solution obtained on reduction with 2·5% sodium amalgam failed to react with Ehrlich's aldehyde reagent but reformed the original yellow pigment on acidification. Dehydrogenating agents such as I_2, $FeCl_3$, and H_2O_2 were without effect.

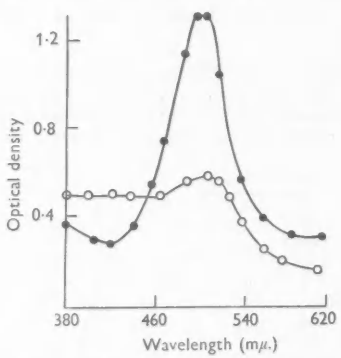

Fig. 8. Effect of pH on the absorption spectrum of the violet pigment. ●—●, pH 1; ○—○, pH > 10.

Changes in absorption spectrum of porphobilinogen solutions at pH 1

HCl (10 N) was added to the porphobilinogen solution, pH 6·8, until the concentration of acid was 7% (w/v) and the pH was less than 1. On standing, the solution became violet and this change was accelerated by sunlight or ultraviolet light. The solution was left to stand until the reaction with *p*-dimethylaminobenzaldehyde was negative. The porphobilinogen concentration and the spectral absorption were determined at 24-hourly intervals. Since neither temperature nor exposure to light was controlled, it was impossible to investigate the order of the reaction. The violet solution possessed an absorption maximum at 503–505 mμ. (Fig. 8) and showed no fluorescence in ultraviolet light. The rate of formation of the violet pigment was directly proportional to the rate of disappearance of the Ehrlich reaction (Fig. 9).

Properties of the violet pigment

The solution gave no fluorescence with zinc acetate and did not react with diazotized sulphanilic acid. Reduction with 2·5% sodium amalgam gave a colourless solution which did not give an Ehrlich reaction but rapidly reformed the violet pigment in acid. The violet solution was unaffected by dehydrogenating agents. When heated at pH 5·2 a violet

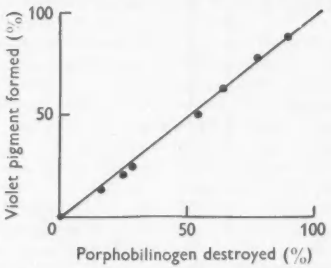

Fig. 9. Disappearance of porphobilinogen with formation of violet pigment. The intensity of the Ehrlich reaction is expressed as the percentage of porphobilinogen destroyed. The absorption at 504 mμ. is expressed as the percentage of violet pigment formed, taking the absorption to be 100% when the Ehrlich reaction had become negative.

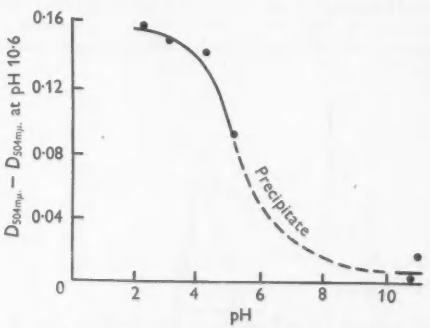

Fig. 10. Spectrophotometric dissociation curve of the violet pigment. $D_{504\ m\mu.}$ = optical density at 504 mμ. at observed pH; $D_{504\ \mu m.}$ at pH 10·6 = optical density at 504 mμ. when the pH was 10·6. This was the lowest optical density recorded.

precipitate was formed which when dissolved in 7% (w/v) HCl showed only a maximum at 503–505 mμ. As the pH of the solution was increased the solution turned red-brown and the optical density at the maximum decreased (Fig. 8). It was not possible to study the dissociation spectrophotometrically as was done with the yellow pigment because a violet precipitate was formed at pH's between 5 and 8, but it appeared that there was a dissociation with a pK of about 5–6 (Fig. 10). The violet precipitate was insoluble in all organic solvents, but after treating with methanolic HCl it was possible to extract the coloured material with $CHCl_3$. The neutral $CHCl_3$ solution gave no fluorescence with zinc acetate. Attempts to crystallize the $CHCl_3$-soluble pigment have so far not been successful.

Attempted purification of the porphyrin formed from porphobilinogen solutions

The solutions obtained after heating various porphobilinogen solutions for 40–60 min. were combined and the porphyrin was adsorbed on to $Ca_3(PO_4)_2$ (Sveinsson, Rimington & Barnes, 1949). The $Ca_3(PO_4)_2$ precipitate was dissolved in methanolic HCl and the ester obtained in $CHCl_3$ solution by the customary methods. The porphyrin was taken up in benzene and purified by adsorption on an alumina column followed by elution with benzene-$CHCl_3$ and $CHCl_3$-methanol mixtures. Insufficient material was obtained for crystallization or for determination of the wavelength of the absorption bands in the visible region. The wavelength of the Soret band of the porphyrin ester in $CHCl_3$ was 405 mμ.

Investigation of a possible ethyl acetate-soluble porphyrin precursor

The method of extraction used was that described by Watson (1951) and Hawkinson & Watson (1952). Freshly passed porphyria urine (500 ml.), having a porphobilinogen content of at least 10 units/ml., was brought to pH 4 with glacial acetic acid and extracted three times with 100 ml. of ethyl acetate. The ethyl acetate extract was washed repeatedly with 2% sodium acetate solution until the ethyl acetate no longer gave any reaction with *p*-dimethylaminobenzaldehyde. It was then washed with 1·5 N-HCl until the acid no longer fluoresced red in ultraviolet light. The ethyl acetate extract was irradiated with ultraviolet light for periods varying from 10 to 60 min., and then extracted with 1·5 N-HCl. In four porphyria urines thus studied the ethyl acetate extracts, even though entirely free of Ehrlich-reacting material, turned pink on irradiation in ultraviolet light but did not show a red fluorescence. The pink 1·5 N-HCl extract of the irradiated ethyl acetate extract showed an absorption maximum at about 490 mμ. but did not fluoresce.

The urine remaining after the ethyl acetate extraction was chromatographed on alumina. The eluate which reacted with *p*-dimethylaminobenzaldehyde was brought to pH 5–6, irradiated with ultraviolet light for 5 min. and subjected to the Sveinsson, Rimington & Barnes $Ca_3(PO_4)_2$ adsorption technique for the removal of any porphyrin which might have been formed. The resulting solution was brought to pH 5·2, heated for 30 min., and was then found to contain porphyrin as judged by a red fluorescence in ultraviolet light and by the presence of absorption in the region 405–410 mμ.

In the four urines studied there was no evidence of the presence of the ethyl acetate-soluble Ehrlich non-reacting precursor. Moreover, porphobilinogen solutions formed porphyrin on heating even though subjected to the procedure alleged by Watson to remove such a precursor.

DISCUSSION

On heating porphobilinogen solutions at pH 5·2 for 5–10 min. a yellow pigment with an absorption maximum at 480 mμ. was formed. On longer heating this absorption maximum decreased with the appearance of the absorption band at 407 mμ. suggesting that porphobilinogen might first give rise to the yellow pigment which was then converted to porphyrin. However, when the chromatographically separated yellow pigment was heated at pH 5·2 the maximum at 480 mμ. decreased, but no porphyrin was formed. It seemed likely therefore that both the yellow pigment and the porphyrin were formed simultaneously, the latter more slowly than the former which was destroyed on prolonged heating.

On standing in acid at pH 1 a violet pigment with a maximum absorption at 503–505 mμ. was formed. It is possible that a porphyrin labile in light was formed during the experiment and was destroyed as fast as it was formed. Whilst pure coproporphyrin and uroporphyrin are stable under these conditions it is known that porphyrins derived from porphyria urines may on occasion be unstable. The appearance of the pigment could be correlated with the disappearance of porphobilinogen, which suggests that this was the only pigment formed in these circumstances. It is possible that the violet pigment was formed in the heating experiments, and its absorption was masked by the greater absorption of the yellow pigment at 480 mμ. When porphobilinogen solutions were heated in 0·1 M buffer, pH 5·2, and the yield of yellow pigment was relatively small (Fig. 3) an absorption band was present in the solution of the precipitate in 0·5 N-HCl with its maximum at 490–495 mμ. However, the supernatants of the porphobilinogen solutions heated in M buffer, pH 5·2, had absorption maxima at 480 mμ. Inspection of the curves in Fig. 6 shows that this shift in the absorption is in the opposite direction to that which would be expected for such a difference in pH. It is more likely that the position of the maximum is due to the presence of a mixture of the yellow and violet pigments.

Porphobilinogen, therefore, appears to give rise to at least three distinct pigments, a porphyrin, a yellow pigment with maximum absorption at 480 mμ. and a violet pigment with maximum absorption at 503–505 mμ., the yields of each depending on the conditions. Porphobilin was described by Waldenström & Vahlquist as a red pigment with an absorption maximum at about 500 mμ., properties in agreement with those found for the violet pigment rather than the yellow pigment.

In the absence of further knowledge it is impossible to do more than speculate upon the nature of porphobilinogen and of the yellow and violet pigments. The inability of these substances to pass from aqueous solutions into organic solvents except after esterification suggests that all have side chains bearing carboxyl groups and also that other hydrophilic groups are unlikely to be present. Monopyrrolic compounds, and compounds containing more than one unconjugated pyrrole ring, e.g. dipyrrylmethanes, the absorption spectra of which

have been examined, have absorption maxima between 250 and 280 mμ. (Pruckner & Dobeneck, 1942). Introduction of ethyl or carbethoxy groups displaces absorption towards longer wavelengths. It is therefore difficult to reconcile the absence of absorption of light of wavelengths greater than 240 mμ. by porphobilinogen with a monopyrrolic or dipyrrylmethane structure unless the pyrrole ring bears some substituent group whose influence on the position of the absorption maximum is opposite to those so far studied.

If it is accepted that porphobilinogen forms porphyrin and the yellow and violet pigments, one α position must carry a potential methyne, —CH=, group the nature and effect on absorption of which is unknown. Pyrrolic substances react with the Ehrlich aldehyde reagent when they have an α position free or bearing a readily displaceable group which must be present if porphobilinogen has a monopyrrolic structure. However, tetrapyrrolic substances such as mesobilirubinogen which do not contain free α positions also react with *p*-dimethylaminobenzaldehyde, and such a tetrapyrrolic structure for porphobilinogen has also to be considered; mesobilirubinogen itself shows no absorption above 250 mμ. (unpublished observation).

Dipyrrylmethenes and other multipyrrolic structures containing conjugated pyrrole rings are all coloured and have prominent absorption maxima in the visible region (Pruckner & Stern, 1937). The yellow pigment may well be a 5:5′-disubstituted dipyrrylmethene, a structure in accord with its absorption spectrum, its green fluorescence with zinc salts, its apparent pK of 9 and its reduction by sodium amalgam to a colourless compound, but there may be additional non-conjugated pyrrole rings linked by methene, —CH_2—, groups, giving tri- or tetra-pyrryl structures (cf. stercobilin (tetrahydromesobilene) or urobilin (mesobilene)). The absorption spectrum of the violet pigment suggests the presence of a more extensive system of double bonds than may be contained in a dipyrryl compound. The absence of a red fluorescence with zinc salts and the failure of sodium amalgam reduction to give a bilane reacting with Ehrlich's reagent, suggests that it is not a biliviolin or bilirhodin type of compound, i.e. a biladiene, biladienone or biladienediol.

Evidence that porphobilinogen gives rise to porphyrin

Porphyrin has been obtained in yields of 0·75–2 μg. per unit of porphobilinogen in four cases of acute porphyria studied. These values agree well with those previously reported (Prunty, 1945; Gray, 1950). Differences in yields are probably explained by minor differences in technique. The maximum yields of porphyrin appear to be obtained on heating in 0·05–0·2N-hydrochloric acid. It is probable that pH 5·2 is optimal for porphyrin formation only when porphyria urines, and not porphobilinogen solutions, are being heated (Grieg *et al.* 1950).

It is difficult to reconcile with the work of Watson and co-workers and of Herbert (1952) our failure to demonstrate an ethyl acetate-soluble Ehrlich non-reacting porphyrin precursor in the cases studied, as well as our failure to obtain a porphobilinogen preparation incapable of forming porphyrin. Neither Watson nor Herbert has indicated the quantities of porphyrin formed from their ethyl acetate-soluble precursors.

Westall (1952) has attempted to separate porphobilinogen from a possible second precursor by paper partition chromatography. Five different solvent systems were used which gave widely differing R_F values for the material reacting with Ehrlich's reagent. On spraying the paper with buffer and heating, porphyrin was found only in those areas of the paper to which the Ehrlich-reacting substance had moved. We have confirmed his results, using a system with butanol saturated with 10% (w/v) acetic acid as the moving phase. Such behaviour supports the view that porphyrin is formed from porphobilinogen unless the porphyrin precursor moves on paper with the same R_F value as porphobilinogen under these conditions. Insufficient material has been available to determine whether porphobilinogen purified by paper partition chromatography can also form the yellow and violet pigments.

It is possible that porphobilinogen is a mixture of two or more closely related chemical substances both reacting with the Ehrlich aldehyde reagent and which react to form porphyrin, yellow and violet pigments. Other precursors of porphyrin may also be excreted by patients with acute porphyria, but no evidence has been found of their presence in the cases we have studied.

SUMMARY

1. The changes occurring when porphobilinogen solutions were (*a*) heated at pH 5·2 and in hydrochloric acid and (*b*) allowed to stand at pH 1, have been studied spectrophotometrically.

2. The spectral absorption at 286–288 mμ. of porphobilinogen solutions as usually prepared has been shown to be due to uric acid.

3. Approximately 2 μg. of porphyrin per unit of porphobilinogen were obtained, the exact yield depending on the conditions of heating.

4. A yellow pigment was formed on heating, which absorbed maximally at 480 mμ. It is suggested that this is a 5:5′-disubstituted dipyrrylmethene or a bilene.

5. On standing at pH 1 a violet pigment of unknown structure was formed which absorbed maximally at 503–505 mμ.

6. It has not been possible to separate a second precursor from porphyria urines.

7. The experiments reported, while not providing conclusive evidence, support the view that porphobilinogen itself can form porphyrin, yellow pigment and violet pigment according to the conditions.

We wish to express our thanks to Prof. C. Rimington for helpful discussions, and to the Medical Research Council for a personal grant to one of us (P. E. B.) and for an expenses grant to C. H. G.

Note. Since this paper was accepted for publication, crystalline porphobilinogen has been prepared from human acute porphyria urine (Westall, R. G. (1952), *Nature, Lond.*, **170**, 614). The results described in the present paper have been confirmed in every respect using crystalline material we have prepared by Westall's method, from the urine of rabbits in which porphyria had been induced by the administration of Sedormid (Schmid, R., quoted by Lowry, P. T., Hawkinson, V. & Watson, C. J. (1952), *Metabolism*, **1**, 149).

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The Ammonia and Urea Excretion of Different Species of Amphibia during their Development and Metamorphosis

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Metamorphosis is a short but none the less critical stage in the development of Amphibia during which extensive changes occur, enabling them to transfer from an aquatic to a more or less terrestrial habitat. The anatomical changes associated with the transition have been described by numerous workers, but the underlying chemical and metabolic transformations demanded by such changes are still largely obscure. The present paper deals with one aspect of nitrogen excretion during metamorphosis.

Some years ago the present author observed that metamorphosis in the common frog was characterized by a change in the chemical form in which nitrogen was excreted. The tadpole, up to the time of metamorphosis, excreted about 10 times more of its waste nitrogen as ammonia than as urea, while the frog excreted nitrogen mainly as urea. The change-over was accompanied by a corresponding increase in the concentration of liver arginase (Munro, 1939).

These changes are interesting because they are in harmony with the concept that the form in which waste nitrogen is excreted is governed by the type of environment, aquatic or terrestrial, within which the animal develops and later functions as an adult. Needham (1931) has collected data on the urinary nitrogen partition of a large number of animal

species. They suggest that aquatic animals in general excrete a much higher proportion of their waste nitrogen as ammonia than do terrestrial species. The reason for this may be that ammonia is a highly alkaline substance and is likely to be damaging to the animal's tissues generally, and to the kidney in particular, unless its concentration in the body is kept low by a copious excretion of urine; which of course is most easily accomplished in a freshwater environment.

There exists among the Amphibia a variety of forms showing different degrees of aquatic and terrestrial adaptation. This is associated with corresponding peculiarities of structure, development and metamorphosis. It thus appeared likely that a survey of the form in which nitrogen was excreted during development and metamorphosis in representative members of the group might provide further and more precise evidence of the relation between this characteristic in an animal and its habitat or manner of development.

MATERIAL AND METHODS

The tadpoles of *Rana temporaria* and of *Bufo bufo bufo* in the stock jars were fed on ant pupae, the axolotls (*Siredon mexicanum*) on worms and chopped liver and the adult *Xenopus laevis* on small pieces of liver. The *Rana* tadpoles were prematurely metamorphosed by immersion for 2 days in a thyroid solution prepared by shaking 1 part of desiccated thyroid gland (B.P.) with 400 parts of water and removing the undissolved material by centrifugation (Huxley, 1925). Metamorphosis was stimulated in the axolotls by injecting 1 mg. thyroxine.

Xenopus eggs were obtained by injecting 10 mg. of a crude extract of anterior pituitary-like hormone (A.P.L.) dissolved in 1 ml. of distilled water into the dorsal lymph sacs of a number of adult male and female *Xenopus* toads. The extract was prepared from human pregnancy urine (Langrebe, 1939). Amplexus occurred some hours later and a batch of about 250 eggs was subsequently obtained. Embryonic development was passed in a dish of water. Hatching occurred in 2–3 days. A week later the larvae began to feed and they were transferred to a tank of water to which had been added an infusion of raffia, mud and weed obtained from a pond supporting a large frog population. More than half of the larvae died during the first week. Only ten survived to the stage of metamorphosis.

Hypophysectomized *Xenopus laevis* adults were obtained as a gift from Prof. Langrebe of Cardiff University to whom the author is indebted.

Each type of experiment varied in certain particulars which are referred to in the appropriate sections, but in every instance the method of determining the nitrogen excretion was to place the living material being examined, eggs, larvae or adults, in a known volume of water for a definite time, usually 24 hr., and then to take samples of the fluid for analysis. In order to prevent diffusion of NH_3 from the water into the atmosphere, the fluid was maintained at pH 6·5 with acetic acid and Na_2HPO_4.

Before being used in an experiment, the animals were fasted for 3 days, in order to reduce contamination of the circumambient fluid with formed excreta. An example will indicate the method of transferring the animals from the stock jars to the experimental containers. A group of twenty tadpoles was removed from the stock jar by suction into a wide-mouthed pipette with a rubber teat. They were then washed with water in a Büchner funnel, gently shaken to drain off the surplus water, and tipped into a dish containing 30 ml. of the buffered fluid and left for 24 hr. in a large glass box where the air temperature was maintained at 18·5° ± 1°.

The technique adopted to collect the nitrogen excretion from the head and gill region of the axolotl separately from that of the posterior region, was similar to that adopted by Smith (1929). The axolotl was placed in a metal box of the type shown in Fig. 1. This is in two parts clamped together

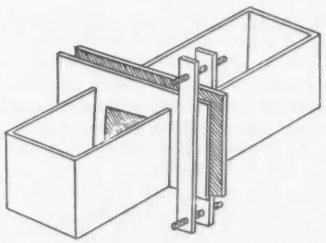

Fig. 1. The two parts of the box used to contain the axolotl. Overall dimensions of box: 18 × 5 × 5 cm.

to provide two compartments. Between them there is fixed a piece of rubber dam with a hole in it sufficiently large to take the body of the animal without unduly compressing it or allowing fluid to leak from the one compartment to the other. Before making an estimation the box was filled with water and the animal placed in the posterior compartment with its head facing the hole in the rubber. A sudden tilt of the box carried the forepart of the animal through the hole and left it invested by the rubber just behind the forelimbs. The water in the box was then drained off, replaced by a measured quantity of buffered fluid (pH 6·5) and the animal left in position undisturbed for some hours.

The NH_3 and urea contents of the samples were determined by the methods described by Conway (1947) using the ordinary Conway diffusion units.

RESULTS

Ammonia and urea excretion during embryonic development

The excretion of ammonia and urea by the developing eggs of *Rana temporaria*, *B. bufo bufo* and *Xenopus laevis* was determined daily until the free-swimming stage. Throughout embryonic development, ammonia formed 80–90% and urea 10–20% of the nitrogen excreted. The total amount of ammonia and urea excreted, however, increased progressively during embryonic development. Expressed as nitrogen excreted/100 eggs/day, it was, at the neurula stage 0·172, 0·046 and 0·046 mg. in *Rana*, *Bufo* and *Xenopus*, respectively, whilst on

Table 1. *The ammonia and urea excretion of the terrestrial toad,* Bufo bufo bufo, *and of the aquatic toad* Xenopus laevis *during normal development and metamorphosis*

Amphibian	Stage of development	Ammonia + urea N excreted/g. body wt./day (mg.)	Amount present as 'free' ammonia (%)
Bufo (adult habitat is terrestrial)	Hindlimbs ⅔ developed	0·147	80
	Hindlimbs functional	0·122	85
	Forelimbs developed under the operculum	0·126	65
	Both forelimbs free. Tailed	0·171	50
	Tail atrophying	0·273	36
	Tail-less	0·251	20
	Adult	0·154	15
Xenopus (adult habitat is aquatic)	No hindlimbs	0·084	85
	Hindlimbs functional	0·087	83
	Both forelimbs free. Tailed	0·084	81
	Tail-less	0·199	77
	Adult	0·091	81

the twelfth day after deposition of the eggs, when embryonic development was complete, it was 0·721, 0·525 and 0·242 mg.

Nitrogen excretion during later larval development and normal metamorphosis

Anurans

Bufo bufo bufo. The proportion of ammonia in the larval excretion remained as high as in the embryonic excretion until the development of the hind-legs. A steady fall then occurred while the hind- and the fore-limbs were growing. Subsequently there was a considerably more rapid fall in the proportion of ammonia excreted, particularly during the stage of tail absorption so that within 5 days it had been reduced from 65 to 20 % (Table 1). At the same time the proportion of urea rose correspondingly. The fully grown toad had only 10–15 % of the waste nitrogen in the form of ammonia.

There was little variation in the sum of the ammonia and urea excreted each day until the stage of intestinal and tail absorption and the freeing of the forelimbs, when it was almost doubled. The rate of nitrogen excretion in the adult toad was, on the other hand, only slightly higher than that of the larva. The changes in the nitrogen excretion during metamorphosis are thus very similar to those occurring in *Rana temporaria* (Munro, 1939).

Xenopus laevis. It was of some interest to compare the nitrogen excretion of an anuran like *Bufo* which leaves the water after metamorphosis with another such as *Xenopus laevis* which undergoes almost as extensive a metamorphosis yet, in spite of its structural adaptation for land life, remains permanently aquatic. Table 1 shows that the nitrogen excretion of this species remains throughout larval development and metamorphosis as ammoniacal as during its embryonic stage. The fully grown adult animal likewise excretes about 80 % of its waste nitrogen as ammonia. Although no change in the proportion of ammonia to urea occurred in the excretion during metamorphosis, the rate at which these two substances were excreted almost doubled during the same period, just as occurred in *Bufo* and *Rana*.

The association of a predominantly ammoniacal excretion and a permanently aquatic habitat in a toad still so well adapted anatomically for land life as *Xenopus*, prompted further investigation, particularly as to the possible occurrence of a seasonal variation in the proportion of ammonia to urea excreted, and to the possibility of inducing experimentally the excretion of a greater proportion of urea. It was not found possible to follow the nitrogen excretion over a whole year, but the ammonia and urea excretion of a group of animals was determined at frequent intervals during 6 months, from February to July. The first part of Table 2 shows that the relative proportions of the two substances did not vary in any consistent way and that the excretion remained predominantly ammoniacal throughout the period.

The possibility of desiccation being able to cause a variation was also considered. Three *Xenopus* toads were exposed to a strong current of dry air for 8 hr. and their body weights thereby reduced 10–20 %. Subsequent determination of their ammonia and urea excretion during the following 3 days failed to show any deviation from the proportions found in untreated animals.

Hypophysectomized Xenopus laevis. The ammonia and urea excretion of three toads, hypophysectomized about 6 months previously, was followed over the same period as for the normal animals referred to above. The proportion of ammonia in the excretion showed no tendency to vary in any consistent way, but it was on the average, considerably lower than that of the normal group (Table 2). From fifteen to

Table 2. *The ammonia and urea excretion of hypophysectomized and unoperated* Xenopus laevis, *determined at intervals over a number of months*

Condition of animals	No. examined	No. of estimations	Ammonia + urea N excreted/g. body wt./day (mg.)	Amount present as 'free' ammonia (%)	Range (%)	Period of observation
Normal	1	23	0·066	82	72–88	Feb.–July
	1	20	0·042	87	70–100	Feb.–July
	1	4	0·084	80	73–86	Feb.–July
	12	10	0·063	80	64–88	Feb.–Apr.
Hypophysectomized 6 months previously	1	25	0·049	54	42–68	Feb.–July
	1	25	0·098	46	30–70	Feb.–July
	1	15	0·114	55	32–70	Feb.–July
Hypophysectomized 3 weeks previously	1	7	0·038	77	67–81	Apr.–July
	1	6	0·045	85	79–94	Apr.–July
	1	11	0·073	76	70–92	Apr.–July
Spayed female	1	11	0·066	74	56–93	Feb.–July

twenty-five determinations of ammonia and urea were made on each toad during the 6 months. In that period the proportion of ammonia averaged 52 % while the corresponding figure for the group of normal animals was 82 %. Determinations were made at the same time of the nitrogen excretion in a spayed female toad and the values found for ammonia and urea were much nearer to the normal than to the hypophysectomized group.

Three more toads were examined at a later date. They differed from the previous animals in having been hypophysectomized for only 3 weeks. Table 2 shows that the proportion of ammonia in the excretion, determined at intervals over a period of 4 months, did not differ much from that found in the group of normal toads. These data diminish the significance of the results with the 'old' hypophysectomized animals. It is difficult, however, to regard the consistently low values for ammonia in the latter group of toads as being within the limits of normal variability. Neither feeding nor starvation produced any marked variation in the proportion of ammonia to urea excreted by this group nor indeed by the other groups, although the total amount of ammonia and urea excreted in these states differed greatly. It is possible that long-standing hypophysectomy may have an influence on the quality of the nitrogen excretion and that the effect is not apparent in more recently operated animals.

Urodeles

Triturus vulgaris *and* Triturus cristatus. The changes in the ammonia and urea excretion during development and metamorphosis in these animals were similar to those occurring in *Rana* and *Bufo* (Table 3). The rate of nitrogen excretion was found to be considerably higher during early larval development than later when body size was much greater.

Axolotl (Siredon mexicanum). Determinations were made of the ammonia and urea excretion of a batch of young axolotls from 1 day after they had hatched until the forelimbs had developed. Another batch of older animals was examined at the same time.

Ammonia formed the greater part of the nitrogen excretion in the earliest stages, but as growth and differentiation proceeded the proportion of waste ammonia fell (Table 4), and during later larval life ammonia and urea were excreted in about equal proportions. One axolotl, the largest, weighing 79 g. was exceptional. It was much darker in colour than the others and the gills, instead of being large and vascular, were small and atrophied. The general appearance was as if metamorphosis had proceeded some way. Table 4 shows that the proportion of ammonia in the waste nitrogen was considerably lower than that of the other axolotls examined.

The rate of nitrogen excretion of the group was distinctly less the heavier the animal up to a body weight of about 10 g. In heavier animals there was no further appreciable decrease.

Nitrogen excretion through the gills of the axolotl. The association of atrophied gills, a larger body weight and a smaller than normal proportion of ammonia in the excretion suggested that the surface area available for excretion might be a factor determining the character of the nitrogen excretion, and that the gills might form part of this excretory surface. Some observations were therefore made on the proportion of ammonia to urea excreted by the gills and head compared with the rest of the body in axolotls with well-developed gills and in others with atrophied gills. Constant watchfulness was required during the course of an experiment for movements of the animal and numerous experiments were vitiated in this way. However, in a number of instances no movements occurred and the results in four such experiments are shown in Table 5.

Table 3. *The ammonia and urea excretion of the newts* Triturus vulgaris *and* T. cristatus *during normal development and metamorphosis*

Amphibian	Stage of development	Body wt. (g.)	Ammonia + urea N excreted/g. body wt./day (mg.)	Amount present as 'free' ammonia (%)
T. vulgaris	Larval	0·08	0·32	75
		0·09	0·21	60
		0·09	0·19	75
		0·12	0·22	74
		0·15	0·15	83
		0·17	0·12	88
		0·20	0·19	63
		0·23	0·07	82
		0·24	0·12	86
	Metamorphosing	0·10	0·23	71
		0·15	0·40	34
		0·20	0·30	40
	Metamorphosed	0·08	0·49	11
	Adult	2·33	0·23	25
		2·50	0·18	11
T. cristatus	Larval	0·42	0·22	69
		0·47	0·27	70
		0·56	0·25	67
	Metamorphosing (gills shrunk)	0·29	0·37	41

Table 4. *The ammonia and urea excretion of axolotls* (Siredon mexicanum) *at different stages during normal development*

Stage of development	Body wt. (g.)	Ammonia + urea N excreted/g. body wt./day (mg.)	Amount present as 'free' ammonia (%)
Hatched 1 day	0·01	0·210	77
Hatched 8 days	0·01	0·367	84
Forelimbs only	0·18	0·169	92
Forelimbs only	0·21	0·210	77
Fore- and hind-limbs	3·70	0·140	70
	4·00	0·074	61
	5·50	0·070	54
	7·50	0·157	36
	10·30	0·066	48
	52·00	0·045	43
	52·00	0·053	60
	55·00	0·035	50
	56·00	0·049	38
Gills shrunken	79·00	0·053	25

The smaller axolotl had well-developed vascular gills, the larger animal had little more than gill stumps remaining. It can be seen from the table that 35 % of the ammonia and urea excreted by the smaller axolotl in 3·5 hr. was lost from the anterior region. On the other hand, only 10 % of the waste nitrogen was excreted by the larger axolotl from the anterior part of its body during a period of 2 hr., in a similar type of experiment. The gills would thus appear, from these observations, to be able to excrete waste nitrogen.

Effect of administering thyroid hormone on the nitrogen excretion

Axolotl. Five axolotls differing in weight from 45 to 70 g. were used for the experiments. Three were injected with 1·0 mg. each of thyroxine and two were used as controls. The ammonia and urea excretion was determined at regular intervals from the time the hormone was administered until after

Table 5. *The ammonia and urea excretion from the head and gills of axolotls determined separately from that of the rest of the body*

Developmental state	Wt. of animal (g.)	Duration of experiment (hr.)	Volume of circumambient fluid (ml.)		Ammonia + urea N excreted (mg.)		Ratio of anterior to posterior excretion
			Anterior	Posterior	Anterior	Posterior	
Larval	10	3·5	25	45	0·054	0·102	0·53
Larval	10	3·5	25	52	0·060	0·105	0·57
Metamorphosing	85	2·0	125	150	0·109	0·602	0·18
Metamorphosing	85	2·0	100	125	0·014	0·437	0·03

metamorphosis was completed, about 25 days later. The results are shown in Fig. 2.

A significant shrinkage in the gills of all the experimental animals could be detected by the tenth day. From about the tenth to the fifteenth day the morphological changes were rapid, and at 25% in the 15 days following the injection of thyroxine, whilst the proportion excreted by the controls showed no consistent change over the same period. About the tenth day after injection the total excretion of ammonia and urea began to increase in the experimental animals and reached

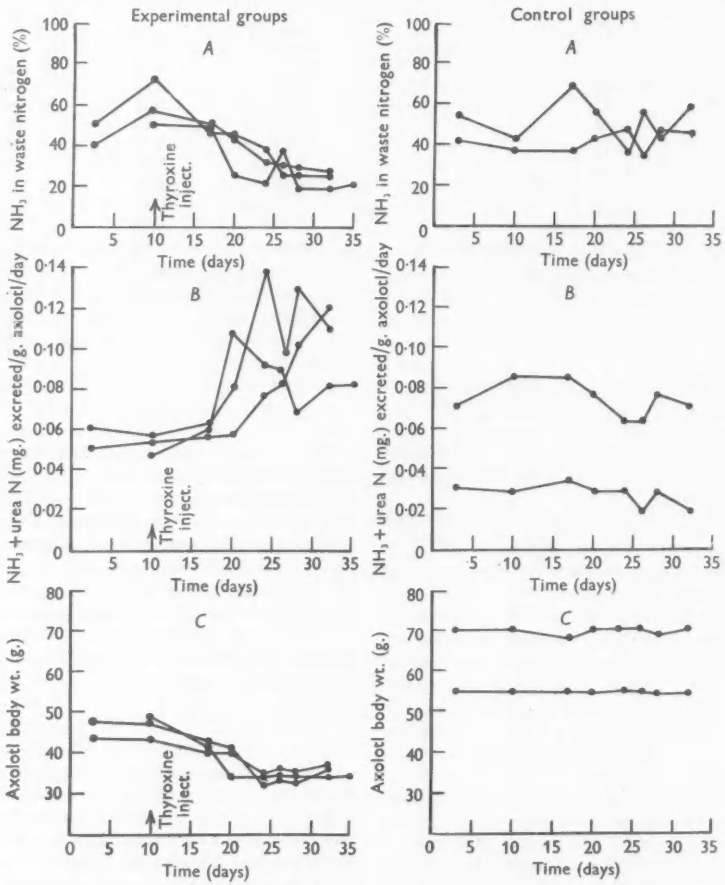

Fig. 2. The changes in axolotls, during thyroxine-induced metamorphosis, in: (*A*) the proportion of ammonia to urea in the waste nitrogen; (*B*) the amount of waste nitrogen (ammonia + urea) excreted/unit wt. of axolotl/day; (*C*) in the body wt. Two control and three experimental animals were used. The latter were injected, each with 1 mg. thyroxine at the time shown by the arrow on the abscissae.

the end of this period the gills were merely stumps and the dorsal fin had been almost completely absorbed. There is a close correspondence between the time of these anatomical changes and of the changes in the character and in the rate of the nitrogen excretion. The proportion of ammonia excreted by the experimental animals fell by about a peak at about the fifteenth day. The body weight, on the other hand, decreased during the same period.

Early larval stages of Rana temporaria. Immersion, in the previously described thyroid solution, of larvae in different stages of development from the tail bud to where the external gills were disappear-

Table 6. *The ammonia and urea excretion of* Rana temporaria *tadpoles at different developmental stages during precocious metamorphosis caused by thyroid extract*

(Tadpoles with hindlimbs $\frac{3}{4}$ developed were immersed in 1:400 thyroid extract for 2 days.)

Time after initial immersion in thyroid solution (days)	Stage of development	Ammonia + urea N excreted/g. body wt./day (mg.)	Amount present as 'free' ammonia (%)
3	Hindlimbs ($\frac{1}{4}$ developed)	0·14	75
5	Hindlimbs ($\frac{3}{4}$ developed)	0·30	66
7	Tail slightly absorbed	0·26	30
8	Tail half absorbed. Forelimbs covered	0·35	35
10	Tail well absorbed. Forelimbs free	0·32	10

ing, had no effect either on their subsequent survival, their rate of nitrogen excretion or the proportion of ammonia to urea excreted. Some acceleration was noted, however, in the speed at which the embryos developed to hatching in the thyroid solutions as compared with the controls.

Later larval stages of R. temporaria. In a typical experiment, thirty tadpoles varying in weight from 100 to 200 mg. were placed for 2 days in the thyroid solution. They were then removed and placed in water without thyroid extract. On the fifth day after the initial immersion one control and six experimental animals had died. On the twelfth day all of the experimental animals, but only three of the control animals, were dead. The visible morphological changes produced by the hormone suggested a precocious metamorphosis differing markedly from the normal process. The tail, for instance, was well absorbed before the forelimbs were freed, and the whole process appeared to be out of phase in comparison with normal metamorphosis. Table 6 shows that changes in the nitrogen excretion accompanied these bodily changes. After immersion in the thyroid solution for 5 days, the total nitrogen excreted as ammonia and urea had doubled and there was a significant diminution in the amount present as ammonia. By the tenth day the nitrogen excretion was mainly in the form of urea.

DISCUSSION

The foregoing survey shows that, when the adult amphibian environment is terrestrial, the waste nitrogen is excreted mainly as urea, when it is completely aquatic, the waste nitrogen is excreted predominantly as ammonia. Likewise during metamorphosis, ammonia excretion is replaced by urea excretion, but only in those circumstances where terrestrial function is assumed as well as terrestrial form. Thus *Xenopus laevis* undergoes almost as extensive a metamorphosis as *Bufo bufo bufo*, yet the proportion of ammonia in the excretion remains as high in the adult as in the larva of *Xenopus*. It also follows that neither the processes of morphological change for terrestrial life, nor those associated with the degeneration of the aquatic organs, can be regarded as responsible for setting in increased motion at metamorphosis the biochemical mechanisms for urea production and excretion.

Development in the urodeles follows a somewhat different course from that of the anurans. There are three pairs of external gills throughout the larval stage. The forelimbs appear earlier than the hindlimbs. When the latter appear the animal has the complete larval configuration and when metamorphosis occurs the only obvious changes are the disappearance of the gills and of the dorsal fin. Compared with other Amphibia the axolotl is unique in that it remains permanently larval and breeds as such, unless stimulated to metamorphose by dry environmental conditions (Chauvin, 1891) or the administration of thyroid hormone.

Although the axolotl, under the stimulus of thyroxine, develops an almost completely ureotelic nitrogen excretion at metamorphosis, it differs, in its later larval stages, from the other Amphibia examined, in excreting a relatively high proportion of its waste nitrogen as urea. It may be that this tendency to ureotelism during the aquatic phase helps to explain the readiness with which the axolotl, as compared with other Amphibia, will metamorphose in response to environmental stimuli which are adverse to continued existence as an aquatic animal, namely, restriction of the environmental water. The more complete the ureotelism, the better prepared is the animal, in one essential respect, for transformation to land life.

Xenopus larvae in the embryonic stage appear to possess distinct urodelan characteristics. After metamorphosis they show not the slightest tendency to leave the water. The adult characters are a queer mixture of the primitive and the highly specialized. The tongueless condition, for instance, is not an archaic feature but an adaptation for the completely aquatic habit. The presence of the hyoid bone, a metamorphic stage and other signs of terrestrial adaptation in the limbs, strongly suggest that

X. laevis was at one time terrestrial and has since undergone a reversion to aquatic life. Why should the ureotelism which most probably characterized the animal when it was living on the land, have now disappeared almost completely? Needham (1931), discussing a somewhat similar problem in regard to uric acid excretion, points out that the heats of combustion of ammonia, urea and uric acid are 91, 153 and 462 Cal. respectively. He comments that no animal would excrete uric acid as its main nitrogenous end-product unless driven to it by the necessity for survival. It would appear that the Amphibia find it correspondingly advantageous, from the evolutionary standpoint, to discriminate, when the environmental conditions permit, between the lesser energy expenditures involved in ammonia and urea production.

X. laevis thus provides an example of a very extensive biochemical reversion associated with little obvious structural reversion. Neither during metamorphosis nor at any other stage of development is there any recapitulation of the phylogenetically earlier ureotelism. There is some evidence, however, that in certain circumstances a significant increase may occur in the proportion of urea excreted by the adult animal. The increased urea excretion was observed in only three toads, all hypophysectomized, but it was consistently present during many months of observation. Whatever the cause of the increase the fact of its occurrence is in itself interesting, since it demonstrates that the evolutionary loss is not absolute and that improvement in the capacity for urea production is again possible.

Alexander & Bellerby (1938) state: 'After the breeding season in July or August the ponds (vleis or pans) in which *Xenopus* live begin to dry up as a result of evaporation and when this process is completed the toads finally aestivate in the mud at the bottom.' This characteristic of *Xenopus* strengthens the belief that a diversion from ammonia to urea excretion can occur, for it is unlikely that any animal excreting ammonia as the main nitrogenous end-product would be able to survive aestivation. The observations made by Smith (1930) on the lung fish (*Protopterus annectens*) are interesting in this connexion. Half of the waste nitrogen was excreted by the active animal in the form of ammonia, but during aestivation all of the waste nitrogen was retained in the tissues as urea. The lung fish thus appears to be able to excrete one or the other form of waste nitrogen as the environmental conditions necessitate. Under the influence of a hormonal stimulation, possibly, *Xenopus laevis* may be able to do likewise.

SUMMARY

1. The ammonia and urea excretion by different types of Amphibia was followed from the embryonic stage through metamorphosis to the adult fully grown stage. The species examined were *Rana temporaria*, *Bufo bufo bufo*, *Xenopus laevis*, *Triturus vulgaris*, *T. cristatus* and the axolotl (*Siredon mexicanum*).

2. Ammonia was the predominating nitrogenous waste product during the embryonic and larval stages of all the species examined. Urea predominated in the post-metamorphic stages of those species which became terrestrial after metamorphosis, while ammonia predominated in the post-metamorphic excretion of *Xenopus laevis*, the only completely aquatic species examined.

3. The metamorphosis of the axolotl (*Siredon mexicanum*), by the administration of thyroxine, resulted in an increase in the amount of urea excreted relative to ammonia. A similar change resulted during the precocious metamorphosis of *Rana temporaria* by thyroid extract.

4. The question of biochemical reversibility in evolution is discussed in relation to the nitrogenous excretion of *Xenopus laevis* which has made a return to the aquatic environment.

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Studies on the Skeletal Tissues

3. THE RATE OF EXCHANGE OF THE INORGANIC PHOSPHATE IN DIFFERENT BONES AND PARTS OF BONES IN VARIOUS SPECIES OF MAMMAL

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Recent work has shown that there is considerable variation in the composition of the mammalian skeleton, according to the origin of the sample of bone and species of animal. In a number of species of animal, the degree of calcification of cleaned trabeculae from the metaphyses of the long bones is lower than that of cortical bone in the region of the middle of the shaft (Weidmann & Rogers, 1950; Rutishauser & Majno, 1951; Perrottet & Duckert, 1951). The degree of calcification varies in different parts of the cortex of the individual long bones from some species (Dallemagne, 1943; Strobino & Farr, 1949) and even greater variations have been shown to occur in cortical bone during growth of animals (Hammett, 1925; Logan, 1935; Burns & Henderson, 1936; Rogers, 1949; Rogers, Weidmann & Parkinson, 1952).

This variability in composition of the skeleton makes it seem likely that there may be similar differences in the rate of local metabolism of the inorganic salts in bones from different species of animal. Earlier studies of the skeleton using isotopes have already shown that the cancellous bones of various species exchange faster than the corresponding cortical bones (Hevesy, 1948). The recognition, however, that the ions of solid calcium phosphate can exchange with these ions in a contiguous liquid phase diverted attention from *in vivo* studies of exchange reactions of the skeleton. Some earlier studies (Hevesy, Levi & Rebbe, 1940; Volker, 1942; Volker & Ginn, 1942), suggested that there may be considerable differences in the rate of exchange of phosphate in different species of animal, but this work is difficult to interpret owing to the use of inadequate numbers of animals and to differences in technique or of expression of results. Very recently, during the course of preparation of this paper, the work of Amprino (1952), Zetterström (1952), Engfeldt, Engström & Zetterström (1952) and Lacroix, Devis & Schicks (1952) has again focused attention on the *in vivo* inorganic salt exchanges in the skeleton.

The present work is an attempt to study the relation between the rate of *in vivo* exchange of phosphate and the type of bone, in different species of animal. It is thought that although ionic exchange between plasma phosphate and bone salts can take place, the operation of other more complicated mechanisms is not precluded.

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METHODS

The isotope was administered to groups of healthy adult animals by intraperitoneal injection of approx. 30 μc./kg. body wt. of a carrier-free solution of $Na_2H^{32}PO_4$ in order to avoid altering the level of blood phosphate. Phenol (0·5%) was added to the stock isotope solution to prevent growth of micro-organisms which otherwise led to local concentrations of ^{32}P.

The measurements of radioactivity were made with a liquid counter using a standard scaling and counting apparatus. The results obtained were corrected for the decay of the isotope during the period between its standardization at the Atomic Energy Research Establishment and its experimental use. The activity of the solution was always checked before injection into animals. The results have been expressed throughout this paper as the following ratio (called specific activity):

$$\frac{(\text{counts/min./mg. P}) \times 10^6}{\text{administered counts/min./kg. body wt.}}.$$

The amount of ^{32}P in the inorganic fraction of the serum of the animals was measured as follows: to 5·0 ml. of serum or plasma from freshly drawn blood (containing 4 mg./ml. NaF) were added 5 ml. of water and 10 ml. of 20% (w/v) trichloroacetic acid (TCA). The precipitate obtained after 24 hr. at 0–4° was removed by centrifugation and the supernatant used for determinations of phosphate and radioactivity.

The bones were dissected, cleaned, powdered samples prepared and analysed by the methods previously described (Weidmann & Rogers, 1950). The same TCA extracts of the powders were used for determinations of radioactivity and phosphate content. This method of estimating ^{32}P in bone samples was compared with the methods used by Manly & Bale (1939) and by Hevesy *et al.* (1940). Both of these groups of workers first removed organic matter from the samples by heating with alkaline glycerol. Manly & Bale (1939) then dissolved the resulting ash in acid and estimated the radioactivity of the solutions. Hevesy *et al.* (1940) reprecipitated the phosphate first as molybdate and then as magnesium ammonium phosphate. The reliability of the method used by us clearly depends upon the completeness of removal of soft tissues, particularly marrow, which contain

Table 1. *A comparison of the present method for the determination of the specific activity of bone samples with those of Manly & Bale* (1939) *and Hevesy* et al. (1940)

Species of animal	Type of bone	Exp. no.	Our method		Method of Manly & Bale		Method of Hevesy *et al.*	
			% P*	Specific activity†	% P*	Specific activity†	% P*	Specific activity†
Cat	Diaphyseal	1	12·0	25	10·8	19	—	—
Cat	Diaphyseal	2	11·8	7	8·1	10	10·9	6
Cat	Diaphyseal	3	12·0	52	11·4	48	10·9	47
Cat	Diaphyseal	4	12·1	37	12·0	33	11·5	39
Cat	Cancellous	3	11·0	400	10·8	360	11·0	410
Cat	Cancellous	4	11·4	300	11·1	300	11·0	310
Cat	Alveolar	1	11·3	54	9·8	54	10·4	47
Rabbit	Diaphyseal	10	12·9	22	9·6	24	—	—

* Measured in dry defatted bone powder.

† Expressed as $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$.

Table 2. *Specific activity of inorganic phosphorus of bones and serum, from various animals, measured 4 hr. after intraperitoneal injection of 30 μc./kg. body wt. of carrier-free* ^{32}P *as* $Na_2H^{32}PO_4$

Species of animal	Specific activity*						Relative activities for series 2 (cancellous bone = 100	
	Series 1			Series 2				
	Cortical bone	Cancellous bone	Serum	Cortical bone	Alveolar bone	Cancellous bone	Cortical bone	Alveolar bone
Rabbit								
No. of animals	13	10	19	5	5	5	12	5
Mean value	42	330	19 600	60	290	450	15	63
S.D.	22	200	7 400	22	130	44	8	28
Cat								
No. of animals	7	7	7	6	6	6	7	6
Mean value	14	240	27 500	14	67	240	6·7	28
S.D.	4	91	13 200	4	21	100	3·5	5
Rat								
No. of animals	13	12	11	7	8	8	14	10
Mean value	46	320	10 900	45	160	310	19	49
S.D.	16	110	3 460	15	76	134	8	14
Ferret								
No. of animals	6	6	6	5	5	5	6	5
Mean value	35	230	15 400	38	99	240	16	42
S.D.	14	60	1 600	12	39	58	5	12

* Measured by the ratio $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$.

highly radioactive compounds, during the preliminary cleaning of the bone. Table 1 shows results for the specific activity of samples examined by our own method in comparison with those obtained using the techniques of Manly & Bale (1939) and Hevesy *et al.* (1940). It will be seen that the measurements made with TCA solutions of the inorganic salts are in satisfactory agreement with those obtained by the other more complicated methods. Only in one instance is the result obtained using our technique higher than is obtained using the other methods—an adequate proof that the method for cleaning bones (Weidmann & Rogers, 1950) does not leave significant amounts of tissues containing radioactive organic phosphorus compounds attached to the bone.

RESULTS

The amount of ^{32}P *appearing in various bones 4 hr. after injection*

Rabbits, cats, rats and ferrets were injected with ^{32}P as previously described and killed 4 hr. later. The femoral cortical and trabecular metaphyseal bones and the mandibular molar alveoli were dissected, prepared and examined by the usual techniques. The results obtained are shown in Table 2. Preliminary inspection of this table shows that the relative activities of the three types of bone

Table 3. *Statistical analysis of the differences between the activities of samples from diaphyseal cancellous and alveolar bones and serum from rabbits, cats, rats and ferrets 4 hr. after the intraperitoneal injection of* ^{32}P

	Cortical bone			Alveolar bone			Cancellous bone			Serum		
	n	*t*	*P*	*n*	*t*	*P*	*n*	*t*	*P*	*n*	*t*	*P*
Rabbit/cat	18	3·28	<0·01	9	4·2	<0·01	15	1·18	0·24	24	1·94	0·07
Rabbit/ferret	17	0·7	0·5	8	2·98	0·017	14	12·8	0·22	23	1·26	0·21
Rabbit/rat	22	0·88	0·41	11	1·97	0·075	20	0·27	>0·5	26	3·02	<0·01
Cat/rat	16	5·85	<0·01	12	2·93	0·013	17	1·26	0·21	14	3·48	<0·01
Cat/ferret	11	3·82	<0·01	9	1·21	0·25	11	0·24	>0·5	11	2·07	0·06
Rat/ferret	15	1·85	0·09	11	1·57	0·15	16	1·86	0·08	13	1·49	0·17

n = degrees of freedom; *t* = Student's statistic; *P* = probability that the groups compared are drawn from identical populations.

are in the same order in all four species of animal. The cancellous trabecular bone is always the most active and the cortical bone is always the least. It is not possible, however, to decide upon the relation between the values for the same type of bone in the different species without the use of significance tests. This is due to the very large variation between individuals of the same groups, a deviation in marked contrast to the close agreement of the results for the chemical composition of bones from different individuals (Weidmann & Rogers, 1950). Statistical tests (Table 3) show that the value for the cortical bone of cats is different from that of rabbits, rats and ferrets ($P < 0·01$), whereas the values for this bone in the latter three species show no differences which can be regarded as significant. The cancellous bones from the four species are all very similar in activity.

The interrelationship between the alveolar bones is more complex. A clear difference is apparent between this bone in the rabbit and the cat. Comparison of the rabbit with the ferret, and the cat with the rat, however, show differences with probabilities of 0·017 and 0·01 respectively, which make it likely that the results belong to different populations but are not sufficiently low to be convincing. Moreover, as has been previously pointed out (Weidmann & Rogers, 1950) it is difficult to be sure that the samples of alveolus are collected from one type of bone only, the lamina dura probably being different from the trabeculae within the alveolus.

The rate of appearance of the tracer in the bone

It had previously been shown (Manly, Hodge & Manly, 1940; Neuman & Riley, 1947) that the amount of radioactive phosphate increases rapidly in the cortical femoral bone of rats for the first few hours after parenteral administration of the isotope. At the same time the level of isotope in the inorganic fraction of the serum drops. After this initial period the amount of ^{32}P in the cortical bone remains constant within experimental error for 18 days and there is only a very slow fall in the serum value during the latter period. Hevesy *et al.* (1940) also found a rapid increase of activity in the cortical bone of rabbits and frogs immediately following the start of a course of frequent injections of the isotope. In this work the authors were attempting to maintain the blood level of ^{32}P constant during the experimental period and using this technique the amount of tracer in the bones continued to increase at a slower rate after the initial rapid rise.

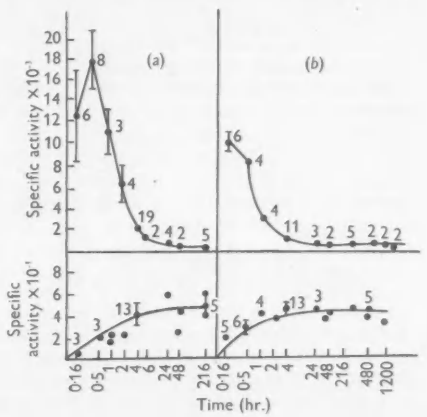

Fig. 1. Variation in the specific activity of the inorganic phosphorus in serum and cortical femoral bone from (*a*) rabbits, (*b*) rats, with time after intraperitoneal injection of carrier-free ^{32}P. Each point represents the mean value obtained for the numbers of animals indicated. The vertical lines represent the standard deviations. Specific activity expressed as

$$\frac{\text{(counts/min./mg. P)} \times 10^{3}}{\text{administered counts/min./kg. body wt.}}.$$

In the present work cats, rabbits and rats were given a single intraperitoneal injection of the isotope. At the time intervals indicated in Fig. 1*a* and *b* and in Table 4, suitable numbers of animals were killed and the isotope concentration in the cortical bones and serum was measured. It will be

seen that the rates of increase of activity in the femoral bones of both of these animals are similar. The only difference between the two species appears in the curves for the activity of the inorganic serum fraction which in the rabbit but not in the rat shows an increase for the first 30–60 min. following injection of the isotope. It is to be presumed that this difference is attributable either to the relative sizes of the two species of animals or to the speeds at which phosphate is absorbed from the peritoneal cavity.

The phosphate metabolism of the cat differs from that of the rabbit and rat as can be seen from Table 4. It has already been shown above that the amount of isotope appearing in the cortical, but not in the cancellous, bone of the cat 4 hr. after injection of the tracer is significantly smaller than for rats, rabbits and ferrets. Table 4 shows that this is probably explained by a slower exchange of the phosphate in the former species. In the cat the amount of ^{32}P in the cortical bone is still increasing 9 days after injection of the tracer, this being the longest period examined. The activity of the serum shows an initial rise for the first 1–2 hr. after injection, followed by a rapid fall during the next 3 hr. During the subsequent experimental period there is a very slight fall in activity. Owing to the cost of cats, and the difficulty in obtaining them, it was impossible to examine sufficient animals to be able to draw a curve for the 9-day period, far from being able to extend the time of the experiment as was clearly desirable. It may be significant that the specific activity of the cortical bone phosphate 9 days after injection agrees with the values obtained for rats and rabbits after 4 hr. Thus it appears that the exchange of phosphate in the cat femoral bone is a very much slower process than in the rabbit or rat and that the smaller amount of ^{32}P found in the cat bone 4 hr. after injection is a reflexion of this and not of the smaller amount of exchangeable ion present in the bone salt.

Table 4. *Influence of time elapsing between intraperitoneal injection of* 30 μc./kg. *body wt.* ^{32}P *as* $Na_2H^{32}PO_4$ *and the specific activity of the inorganic* P *in the femoral cortical bone of the cat and rabbit*

	Cat		Rabbit	
Time	Mean specific activity*	No. of animals	Mean specific activity*	No. of animals
10 min.	—	—	6·3	3
30 min.	10	1	22	3
1 hr.	—	—	25	2
4 hr.	14	7	42	13
2 days	12	2	35	2
9 days	61	4	45	5

* Expressed as

$$\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$$

The amount of ^{32}P appearing in different parts of shaft bone

The results of Manly *et al.* (1940) and of Neuman & Riley (1947) for rats have been confirmed, showing that some fraction of the bone salts appears to exchange faster than the remainder and experiments with rabbits have yielded similar results. The former authors suggested that there is a labile fraction which amounts to about one-twelfth of the salts in cortical bone, and to about one-sixth in the epiphysis. The initial period of rapid uptake has also been interpreted (cf. Hevesy, 1948) as being due to exchange between the plasma and the ions on the surface of the bone salt crystals. Very recently Zetterström (1952) has used saturated ammonium sulphate and acetate buffers to extract fractions of high activity from bone obtained from animals treated with ^{32}P.

In the present study the activities of samples of bone powder from three arbitrarily selected regions of the shaft bone have been examined. These regions are (*a*) a thin layer immediately next to the marrow, (*b*) a thin layer immediately under the periosteum and (*c*) a layer from the 'middle' of the cortical bone. All the samples were taken from the region of the longitudinal mid-point of the femur and tibia of rabbits. If the period of rapid initial uptake is to be regarded as a saturation of the surface of all the crystals within the shaft we might expect the activities of the three samples examined to be similar. If, however, it corresponds to the rapid exchange of some small fraction of the bone salt due to either greater competence of blood circulation, or to the localization of some biological exchange mechanism in cells concentrated in this region, then the samples might show considerable differences in activity.

Table 5 shows the results obtained for the activities of these three samples taken from bones of animals killed at various periods after dosage with the tracer. It will be seen that the layer bordering on the marrow has a very much higher activity than the other two layers. In the animals examined 4 hr. after receiving the tracer the activity of the inner bone layer is 5–7 times that of the layer from immediately under the periosteum. As the period between injection of the tracer and examination of the bone extends, the very large difference tends to decrease. As might be expected with such arbitrarily selected regions there is even greater variation from individual to individual than when pooled samples of shaft bone are examined. Owing to these variations it is not possible to follow the course of activity changes with time after injection of tracer.

Table 5. *The specific activity of selected samples from cortical bone of adult rabbits. Sample (a) from the sub-periosteal layer; (b) from the 'middle' layer; (c) from the layer next to the marrow*

Time after intraperitoneal injection of ^{32}P (hr.)		Specific activity*					
		Femur			Tibia		
		a	*b*	*c*	*a*	*b*	*c*
4	Mean	75	58	520	72	68	350
	No. of animals	5	5	5	5	5	5
	Range	44–120	37–100	269–770	44–130	35–110	173–670
24	Mean	40	29	100	40	30	120
	No. of animals	6	6	6	5	6	6
	Range	9–69	11–58	25–280	15–86	10–49	31–200
48	Mean	45	32	150	74	49	170
	No. of animals	6	6	6	6	6	6
	Range	18–72	23–45	63–330	16–170	17–97	52–370
216	Mean	28	24	92	35	30	72
	No. of animals	3	4	4	4	4	4
	Range	17–38	8–48	48–330	20–47	12–50	49–97

* Expressed as $\frac{(\text{counts/min./mg. P}) \times 10^8}{\text{administered counts/min./kg. body wt.}}$.

It is thought, moreover, that to attempt to do this by simply increasing the number of animals used would be unnecessarily cumbersome and that it is better to seek for some more exact method of sampling, or for some other way of examining the distribution of tracer within the bone. The activity of the layer next to the marrow remains higher than that for the pooled sample from the whole shaft, throughout the period of the experiment. The other two layers examined do not differ from the value for the pooled samples. These results are in good agreement with the very recent work of Engfeldt *et al.* (1952) whose autoradiographic pictures suggest qualitatively that the greatest density of ^{32}P is in the Haversian systems close to the marrow. Thus results based on an examination of samples of the whole shaft give an erroneous picture and the apparently steady state in the activity of the diaphyseal bone achieved 4 hr. after the injection of tracer may represent only the smoothing of a complicated pattern of internal fluctuations.

It may be noted that if the concentration of ^{32}P in the trabeculae (cf. Table 2) is compared with that in the inner layer of the shaft 4 hr. after injection of the isotope, the great difference which has hitherto always been found between cortical and cancellous bone disappears.

DISCUSSION

The work presented here together with that of Engfeldt *et al.* (1952), Amprino (1952) and Lacroix *et al.* (1952) emphasizes the degree to which the rates of exchange of inorganic phosphate between plasma and bone salt vary from one region of a bone to another and from one species of animal to another. The inner layer of the shaft preferentially accumulates ^{32}P and Engfeldt *et al.* (1952) have shown that tracer localizes in the poorly calcified young osteons. It is known (Rogers *et al.* 1952) that the inner layer of shaft bone in some species of animal is relatively poorly calcified, and is therefore presumably rich in young osteons. These results might all be explained by assuming either differences in local blood supply, greater diffusion through freshly deposited bone, or the initial deposition of bone salt in some form which subsequently changes. The latter assumption would seem to be necessary to explain Amprino's (1952) observation that areas taking up large amounts of tracer *in vivo* also do so *in vitro* even after incineration. This assumption alone, however, seems unlikely to be sufficient to explain the finding (Neuman & Mulryan, 1950) that the amount of phosphate exchanged by young fresh periosteal bone *in vitro* is much greater than that by ash produced from the powder by treatment with alkaline glycerol; the extent of exchange by fresh bone powder is far greater than could be accounted for by superficial ions, is partially irreversible, and much affected by temperature. The authors explain their results as being due to 'recrystallization' of the bone salt. If, as seems probable, the most rapid exchange of phosphate takes place in forming osteons, even in adult animals, it seems reasonable to consider the possibility that, in addition to the direct exchange of plasma and bone salt ions, enzymic mechanisms of the type already involved in explaining the phenomena of calcification in young animals, may also be involved in controlling the rate of exchange of phosphate in adults.

The present results call attention to the danger of drawing conclusions from results for the exchange of

inorganic ions obtained using samples representing any considerable volume of bone. Many of the earlier results for the rate of exchange of the inorganic constituents of the skeleton represent only mean values for a series of areas in different states of equilibrium with the plasma.

SUMMARY

1. The rate of exchange of phosphate in the cortical bone of cats is very much slower than in this type of bone in rabbits and rats. This difference does not extend to the trabeculae of the cancellous metaphyseal bones.

2. The distribution of the tracer within the femoral and tibial cortical bone of rabbits shows a high degree of heterogeneity. The layer next to the marrow is always very active compared with the rest of the cortex. The outer layers (sub-periosteal) have a relatively low activity but are slightly higher than those from the depth of the bone.

3. The significance of this heterogeneity of distribution is discussed.

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Bromide Determination in Body Fluids

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In a research involving the distribution of administered bromide between blood plasma and cerebrospinal fluid (c.s.f.), a method applicable to samples of about 1 ml. fluid containing from about 5 to 50 mg. bromine/100 ml. was required. The long-known colour reaction between bromide and auric chloride, used by Walter (1929), Wuth (1927) and others, was regarded as insufficiently accurate for our purposes. A number of methods depending on other principles seemed to be too elaborate or insufficiently proved.

A method based on the Van der Meulen (1931) reaction: $Br^- + 3OCl^- \rightarrow BrO_3^- + 3Cl^-$ has proved to be suitable in most cases, and a preliminary report of this has already been given (Hunter, 1952). This principle was used by Dixon (1934) to determine bromide in normal blood, using 10 ml. The reaction was notably improved by D'Ans & Höfer (1934) who used phosphate instead of borate as buffer, and formate, instead of hydrogen peroxide, to destroy excess of hypochlorite. The reaction was further studied by Kolthoff & Yutzy (1937), and Friedman (1942) has used it to determine bromide in trichloroacetic acid filtrates from blood.

The present communication is based on the above principles, but the procedure has been modified and

the pretreatment of the material to be analysed has been described, with a view to giving increased speed and accuracy to the method.

EXPERIMENTAL

Reagents. 40% (w/v) $NaH_2PO_4.2H_2O$; sodium hypochlorite, 1·0 N in 0·15 N-NaOH; sodium formate, 50% (w/v); sulphuric acid, 6 N; potassium iodide, 20% (w/v); starch, 0·2% (w/v); sodium thiosulphate, 0·005 N; potassium hydroxide, 2·0 N; potassium carbonate, 2·0 N; ethanol, absolute.

No great precautions are necessary to avoid the presence of bromide in most reagents; very little seems to be present in most laboratory materials, except HCl and halides.

Method for simple solutions

The following has been found suitable for 50–350 μg. Br. To a solution of bromide (about 6 ml.) in a 100 ml. Erlenmeyer flask, are added 5·0 ml. phosphate buffer, followed by 3·0 ml. hypochlorite solution. The flask is then immersed in a boiling-water bath for 10 min. The flask is removed and 1·0 ml. formate solution is added. The flask is well shaken, and immersed again in the boiling-water bath for 5 min. The flask is then cooled in water and 5·0 ml. 6 N-H_2SO_4 are added, followed by iodide solution (1·0 ml.). The solution is immediately titrated with 0·005 N-$Na_2S_2O_3$ using six drops of 0·2% starch solution as indicator. A blank determination is carried out at the same time, omitting the addition of bromide:

1 ml. 0·005 N-$Na_2S_2O_3 \equiv 66{\cdot}7$ μg. Br.

Notes

(1) The aqueous volume at start should not exceed about 10 ml. When the volume was 25 ml., only about 80% of the Br present was recovered.

(2) Addition of up to 5 ml. 2 N-NaOH has no effect on the results.

(3) Increasing the phosphate to 11·0 ml. has no effect on the results.

(4) One ml. of the hypochlorite solution is insufficient; with this amount less than 80% Br present is accounted for. If 6·0 ml. hypochlorite solution are used correct values are obtained, but the blank value is thus unnecessarily increased. A blank titration is indispensable in this method as a small amount of ClO_3^- is always present in the reagent. A rather lower blank has been found with a reagent made from fresh specimens of a commercial bleach called Parozone (6 vol. + 4 vol. water) than with the reagent as usually made by passing Cl_2 into cold NaOH solution. The blank in the present procedure, using diluted Parozone, is usually less than 0·20 ml. of 0·005 N-thiosulphate. This hypochlorite solution is stable for many months if stored in a refrigerator.

(5) One ml. formate solution is ample to destroy the excess hypochlorite.

(6) Five minutes' heating is insufficient to convert quantitatively the bromide to bromate.

(7) A carrier to hold nine flasks which can be immersed in a 26 cm. water bath has been found convenient.

Results

With this method bromide present can be quite accurately accounted for, as shown in Table 1.

Table 1. *Determination of bromide in simple solution*

Br (μg.)	
Present	Found
320	319·6, 319·0, 319·0
160	161·4, 159·0, 160·5
50	50·6, 50·8, 49·7
10	10·6, 10·2, 10·0

Chlorides, even in considerable amount, have little effect on the method, and this is one of its great advantages. Indeed, Van der Meulen added NaCl to complete the reaction, but Kolthoff & Yutzy showed this to be unnecessary. This subject has been exhaustively studied by Haslam & Moses (1950) who used the method to determine Br in brines. The effect of varying concentrations of bromide-free NaCl on the blank values and with Br levels at 10 and 50 μg. was tested as shown in Table 2.

Table 2. *Effect of* NaCl *on blank titrations and on values using small amounts of bromide*

NaCl added (mg.)	Blank (ml. 0·005 N)	Br (μg.) Present	Br (μg.) Found
0·0	0·18	10·0	10·0
		50·0	50·6
30·0	0·19	10·0	10·6
		50·0	50·6
60·0	0·18	10·0	10·0
		50·0	50·0
90·0	0·18	10·0	10·0
		50·0	49·2
500·0	0·16	50·0	39·3
1000·0	0·16	50·0	30·7
2000·0	0·17	50·0	29·3

On the other hand, iodide titrates quantitatively with the method. A commercial specimen of KI, which contained a small amount of Br, showed the following values: 103·6, 212·4, 314·0 and 419·6. (Present 100, 200, 300 and 400 μg. as I respectively.)

Application of method to body fluids

Some workers have used protein-free filtrates of blood directly for bromide estimation by this method, although one might expect interference through the presence of urea, phenols and other organic substances. Trichloroacetic acid filtrates of blood have been tried here and sometimes found to give nearly correct values, but gross inaccuracies have also been encountered. Results with ashed and unashed urine, shown in Table 3, illustrate this point. Accordingly, all bromide determinations on body fluids have been preceded by ashing.

The ashing of more than 0·1–0·2 ml. of blood or serum is tedious and if none of the bromide in blood is in organic combination a filtrate may be used. It should be such that it may be readily dried and ashed. Neither tungstic acid nor trichloroacetic acid seemed suitable for this, and as a strictly protein-free filtrate is not necessary, a procedure similar to that used by Conway & Flood (1936) has been adopted. The use of absolute ethanol, instead of 95% methanol, permits less dilution of sera. The procedure for deproteinization and ashing of the filtrate is as follows.

Table 3. *Effect of ashing on the determination of* Br *in urine*

(1 ml. of urine taken in each determination.)

Urine no.	Br added (μg./ml.)	Ashed: Br found (μg./ml.)	Ashed: Error (%)	Not ashed: Br found (μg./ml.)	Not ashed: Error (%)
1	0	3	—	5	—
	100	102	−1	93	−12
	300	306	+1	278	− 9
2	0	4	—	82	—
	100	100	−4	88	−52
	300	292	−4	81	−79
3	0	5	—	16	—
	100	102	−3	59	−49
	300	298	−2	166	−47

Table 4. *Recovery of bromide added to serum and to serum ethanol filtrates*

(To 3·0 ml. normal serum 2·4 mg. Br were added in 0·24 ml. solution. In other experiments, to the ethanol filtrate from 0·5 ml. original serum, 360 μg. Br were added. In all cases 0·5 ml. solution was taken. All filtrates were dried and ignited in nickel crucibles and determinations completed as described in text. Values given are μg. Br in 0·5 ml.)

Material	No. of detmns.	Br added	Br present	Br found: Mean	Br found: S.D.	Br found: Range
Serum	1	0	2·9	—	—	—
Serum + bromide	6	370·3	373·2	370·9	2·2	367·1–373·8
Serum filtrate + bromide	6	360·0	362·9	360·5	1·8	359·0–363·7

To 4·0 ml. absolute ethanol in a centrifuge tube is added dropwise 1·0 ml. serum. (If less than 1 ml. is available the difference is made up with ethanol.) The contents are thoroughly mixed by rapid rotation of the tube between the hands. The contents are centrifuged, and 4·0 ml. of the supernatant fluid are transferred to a 30 ml. nickel crucible to which has been added 1 drop each of 2·0 N-KOH and 2·0 N-K_2CO_3. The crucible is placed in an oven at 110° to dry.

With c.s.f. and urine the preliminary treatment with ethanol is usually not required, the necessary amounts of fluid being taken directly into the crucible with the small amount of fusion mixture.

The ashing is carried out with a half-ventilated ordinary bunsen flame, with the crucible on a pipe-clay triangle. The flame is directed downwards around the outside of the crucible successively at the corners of the triangle until patches of dull red appear. It is then directed symmetrically around the bottom of the crucible. The contents melt slightly as the crucible begins to redden. When a dull red has spread all over the bottom, the flame is removed and the crucible is transferred to a metal plate to cool. The whole procedure occupies little over a minute. There is no appreciable loss of bromide by this procedure; the presence of a little carbon has no harmful effect.

The contents of the crucible are now transferred to a 100 ml. Erlenmeyer flask as follows. With a pipette about 3 ml. water are run down the sides of the crucible. The material is stirred with a small glass rod and transferred to the flask. The process is repeated with about 2 ml. water, then 1 ml. water. To the flask are now added 5 ml. phosphate solution, and 3 ml. hypochlorite reagent and the procedure continued as described for simple bromide solutions.

Bromide added to blood sera is adequately accounted for as shown in Table 4.

DISCUSSION

The presence of large amounts of sodium chloride has a tendency to lower the blank value, but the effect is slight. The addition of up to 90 mg. has no appreciable effect on bromine values of 10 μg. but the addition of 500 mg. sodium chloride or more lowers the values seriously even at the 50 μg. level. With biological material the sodium chloride originally present could exceed 90 mg. only when relatively large amounts of material like blood and urine, were taken. Indeed, any chloride present in biological material is likely to be rather irrelevant where the hypochlorite reagent contains at least 1 % sodium chloride, and where Cl^- is a product of the main reaction. Nevertheless, when bromide values are low and the sodium chloride present exceeds 100 mg., a calibration curve showing the effect of chloride on the bromide levels in question should be constructed.

The fact that iodide is determined by this method does not invalidate it for present purposes, as the amount of iodide normally present in blood is negligible. However, in the use of the method with material from hospital patients, one should be aware of interference that may arise from iodide medication. Relatively large amounts of iodide are found not uncommonly in urine.

Determination of bromide by this method is accurate to about ± 1 %, when the bromine present is greater than about 10 mg./100 ml. and when

0·5–1·0 ml. is taken. With bromine values from 3 to 10 mg./100 ml. the error is within ±5%. A minimum of about 50 μg. bromine is desirable with the method, although with special care, fair estimates may be made with considerably less. Many hundreds of determinations have been performed with this method and it has been found very reliable and trouble-free. It is well adapted to the determination of bromine in many samples in a short time.

The method has also been used with whole blood and erythrocytes. In the latter case, haemolysis with an equal volume or 2 vol. of water has preceded precipitation. In the presence of large amounts of protein, methanol yields a more discrete precipitate and may be substituted for ethanol. If whole erythrocytes or haemoglobin solutions are ashed, the potential interference of ferric ions in freeing iodine from iodide should be borne in mind. However, the concentration of phosphate present under the conditions described adequately suppresses this action.

SUMMARY

1. Details are given for the application of the Van der Meulen reaction—the oxidation of bromide to bromate by hypochlorite—to the determination of bromide in blood, cerebrospinal fluid and urine.

2. The method is not affected by the presence of chloride in moderate concentration, but iodide reacts quantitatively like bromide.

3. This semi-micro method is well adapted to the determination of bromide in body fluids following bromide administration.

4. The accuracy is about ±1% for samples containing 50 μg. bromide in 0·5 ml. fluid.

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A Note on the Hydrolysis of the Urinary Conjugated Adrenocortical Steroids by β-Glucuronidase Preparations

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It was reported by Kinsella, Doisy & Glick (1950) that the yields of 'reducing lipids' extractable from the urine of human subjects by a chloroform-ether mixture could be increased 3- to 5-fold by a preliminary incubation of the urine with an enzyme preparation obtained from *Escherichia coli* which was rich in β-glucuronidase activity. At about the same time Corcoran, Page & Dustan (1950) reported 10- to 20-fold increases in extractable formaldehydogenic and glycogenic material after incubation of urine with a commercial preparation of β-glucuronidase. Shortly afterwards it was independently observed by Cox & Marrian (1951) that the yield of chloroform-extractable 'formaldehydogenic steroid' obtainable from urine could be increased about tenfold, compared with that obtained after hydrolysis at pH 1·0 for 24 hr., by incubating the urine, or butanol-extractable material from urine, with ox-spleen β-glucuronidase preparations. Following these pioneering studies, preliminary treatment of urine with bacterial or animal-tissue β-glucuronidase preparations has been widely used in quantitative studies on the urinary glycogenic, reducing, and formaldehydogenic steroids, as well as in work directed towards the isolation and characterization of such steroids (cf. Venning, 1951, 1952; Kinsella, Baggett & Glick, 1951; Daughaday, Farr & Houghton, 1951; Corcoran, Dustan & Page, 1951; Cohen, 1951; Baggett, Glick & Kinsella, 1952; Schneider, 1952; Romanoff, Wolf & Pincus, 1952; Wilson, 1952; Bayliss, 1952). Cox (1952) has also recently shown that the yield of acetaldehydogenic material, determined by periodate oxidation, which can be

extracted from urine with chloroform can be significantly increased by a preliminary treatment of the urine with β-glucuronidase preparations.

Although it seemed probable that the increased yields reported in the literature of extractable 'adrenocortical steroids' following incubation with β-glucuronidase preparations were largely or wholly due to hydrolysis of urinary steroid glucuronides by β-glucuronidase, it was considered by the present authors that it would be unwise to assume that this was necessarily the case in view of the relative crudeness of the enzyme preparations used and the conspicuous absence of adequate controls in most of the published experimental work. Thus the possibility could not be ignored that the liberation of free steroids might have been due to the hydrolysis of steroid conjugates other than glucuronides by an enzyme in the bacterial or tissue preparation other than β-glucuronidase. Furthermore, the possibility also had to be considered that the observed increased yields of reducing, formaldehydogenic or acetaldehydogenic material might have been the result of hydrolysis by enzymic action of precursors in the enzyme preparation itself: e.g. the liberation of glycerol (formaldehydogenic) from incompletely defatted enzymic preparations by lipase action.

These possibilities have now been investigated in a series of experiments in which the chloroform-extractable formaldehydogenic and acetaldehydogenic substances were determined in the butanol-extractable material from the urine of normal men after incubation with ox-spleen β-glucuronidase (*A*), and the values compared with those obtained in control experiments in which the urine extracts were incubated with boiled enzyme (*B*), in which the enzyme was incubated alone (*C*), and in which the extracts were incubated with the enzyme in the presence of saccharate (*D*).

EXPERIMENTAL

β-Glucuronidase preparations. Ox-spleen extracts were prepared and standardized for β-glucuronidase activity with phenylglucuronide as described by Cox (1952).

Collection and extraction of urine. Four 24 hr. urine specimens were collected from normal men using 100 ml. butanol as a preservative for each. The pH of each specimen was roughly adjusted to 7 by stirring in 50 g. $NaHCO_3$, and the urine was then extracted three times with equal volumes of butanol. The extract was evaporated under reduced pressure in the water bath, the last traces of butanol being removed by azeotropic distillation under reduced pressure after the addition of ethanol. In order to remove the greater part of the free unconjugated steroids from these residues they were washed three times with 400 ml. portions of dry boiling $CHCl_3$.

Incubations. Each residue, prepared as described above, was dissolved in 300 ml. water and 30 ml. M-acetate buffer (pH 4·5) added to the solution. After adjustment to pH 4·5 with acetic acid the solution was made up to 400 ml. with water and divided into three equal portions. The undermentioned mixtures were then made up and incubated at 37° for 48 hr. after the addition of 15 ml. $CHCl_3$.

A, 150 ml. urine extract; 15 ml. water; 5000 glucuronidase units.

B, 150 ml. urine extract; 15 ml. water; 5000 glucuronidase units (boiled).

C, 155 ml. water; 10 ml. M-acetate buffer (pH 4·5); 5000 glucuronidase units.

D, 150 ml. urine extract; 15 ml. 0·1 M-saccharate (adjusted to pH 4·5); 5000 glucuronidase units.

After the completion of the incubation, each mixture was extracted three times with 200 ml. portions of $CHCl_3$. The $CHCl_3$ extract was washed once with 200 ml. 0·1 N-NaOH and three times with 100 ml. volumes of water. The alkali washing and the first two water washings were back-extracted with 100, 50 and 50 ml. respectively of $CHCl_3$, and each back-extract added to the main bulk of $CHCl_3$ before proceeding with the next washing. The washed $CHCl_3$ extract was dried by standing over Na_2SO_4 (20 g.) for 20 min., filtered, and evaporated to dryness under reduced pressure.

Table 1. *Liberation of formaldehydogenic substances (FS) from urine extracts by incubation with ox-spleen β-glucuronidase preparations*

(*A*, extract + enzyme; *B*, extract + boiled enzyme; *C*, enzyme blank; *D*, extract + enzyme + saccharate.)

Urine batch no.	FS (as mg. HCHO/24 hr. urine specimen)			
	A	*B*	*C*	*D*
3	0·690	0·093	0·048	0·101
	0·672	0·104	0·032	0·109
4	0·846	0·099	0·060	0·139
	0·786	0·116	0·053	0·149
6	0·401	0·020	0·023	0·066
	0·451	0·035	0·007	0·060
8	0·922	0·129	0·083	0·192
	0·942	0·123	0·070	0·202

Table 2. *Liberation of acetaldehydogenic substances (AS) from urine extracts by incubation with ox-spleen β-glucuronidase preparations*

(*A*, extract + enzyme; *B*, extract + boiled enzyme; *C*, enzyme blank; *D*, extract + enzyme + saccharate.)

Urine batch no.	AS (as mg. CH_3CHO/24 hr. urine specimen)			
	A	*B*	*C*	*D*
3	0·482	0·092	0·013	0·077
	0·485	0·089	0·010	0·089
4	0·422	0·069	0·013	0·076
	0·443	0·069	0·025	0·063
6	0·253	0·021	0·000	0·025
	0·245	0·023	0·008	0·032
8	0·319	0·044	0·034	0·061
	0·335	0·042	0·027	0·057

Determinations in duplicate of formaldehydogenic and acetaldehydogenic substances were carried out on the residue by the method of Cox (1952). The results of experiments on four separate 24 hr. urine specimens are shown in Tables 1 and 2.

DISCUSSION

On comparing the values obtained from the series *A* incubations with those obtained from the boiled enzyme controls (*B*) it will be seen that the enzymic hydrolysis increased the yields of extractable formaldehydogenic and acetaldehydogenic substances about 7- to 15-fold and 5- to 11-fold respectively. The amounts of formaldehydogenic and of acetaldehydogenic substances determined in the enzyme blank series (*C*) were about half or less than half of those determined in the boiled enzyme controls (*B*), showing that the amounts of extractable formaldehydogenic and acetaldehydogenic substances liberated during the incubations from precursors in the enzyme preparations were of no quantitative significance. By comparing the values obtained in series *D* with those in series *A* and *B* it can be seen that saccharate largely, but not completely, inhibited the liberation of extractable formaldehydogenic and acetaldehydogenic substances by the enzyme preparation. Since Karunairatnam & Levvy (1949) showed that saccharate is a powerful inhibitor of β-glucuronidase, this constitutes strong evidence that the observed liberation of formaldehydogenic and acetaldehydogenic substances involves the hydrolysis of glucuronides by β-glucuronidase. (Levvy (1952) has shown that the inhibiting action of saccharate solutions upon β-glucuronidase activity is due to the presence of saccharo-1:4-lactone.) Since the values obtained in series *D* are slightly higher than those in the boiled enzyme controls (*B*) it is possible that enzymic hydrolysis of some other type of conjugate may also be involved to a small extent in the liberation of formaldehydogenic and acetaldehydogenic substances. It seems more probable, however, that the concentration of saccharate employed was not sufficiently high to inhibit β-glucuronidase activity entirely.

It should be pointed out that the evidence presented here does not exclude the possibility that the observed increases in extractable formaldehydogenic and acetaldehydogenic substances resulting from incubation of urine extracts with β-glucuronidase preparations may be partly due to the hydrolysis of glucuronides of chloroform-extractable formaldehydogenic and acetaldehydogenic substances other than steroids.

SUMMARY

1. The liberation of chloroform-extractable formaldehydogenic and acetaldehydogenic substances on incubation of urine extracts with ox-spleen preparations rich in β-glucuronidase activity has been studied.

2. Control incubations with enzyme preparations but no urine extract have shown that substantially all of the formaldehydogenic and acetaldehydogenic substances liberated arise from precursors in the urine and not by the enzymic decomposition of precursors in the spleen preparation itself.

3. The liberation of formaldehydogenic and acetaldehydogenic substances from urine extracts by incubation with ox-spleen preparations is largely inhibited in the presence of 8×10^{-3}M-saccharate. This provides strong support for the view that this liberation is due to the hydrolysis of glucuronic acid conjugates by β-glucuronidase.

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A Modified Procedure for the Microdetermination of Citric Acid

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During the course of an investigation of the citric acid content of bones and teeth it became necessary to determine amounts of the acid varying from 20 to 100 μg. The method of Weil-Malherbe & Bone (1949) was employed and found to be very reliable for 0·2–1·0 mg. citric acid, but with smaller amounts the results were very erratic and recoveries were low and variable. A detailed study of the method applied to the estimation of quantities up to 100 μg. was undertaken as a result of which certain modifications are suggested to increase the sensitivity and precision of the method, while at the same time giving increased speed and simplicity of working. The modified technique applies equally well to the determination of larger quantities of citric acid, up to 1 mg.

The method of Weil-Malherbe & Bone is based on the oxidation of citric acid to acetonedicarboxylic acid in the presence of sulphuric acid and the conversion of this compound to pentabromoacetone. The most important respect in which it differs from earlier methods is in the use of vanadic acid as oxidizing agent instead of permanganate. Bromination of the acetonedicarboxylic acid is effected by bromine water, excess of which is removed with sodium thiosulphate. The pentabromoacetone is then extracted with light petroleum, a portion of which is shaken with sodium sulphide solution. The intensity of the yellow colour produced in the aqueous phase is measured with a suitable photoelectric instrument.

The source of error was eventually traced to an excess of thiosulphate, and, in view of the impossibility of avoiding a local excess, it was decided to use another reducing agent. Ferrous sulphate, recommended by Pucher, Sherman & Vickery (1936) and by Taussky (1949) was finally chosen because a slight excess of this reagent does not interfere. A bromide-bromate mixture was substituted for saturated bromine water and the ammonium vanadate incorporated in this solution.

In the original method the tubes containing the citric acid solution are cooled to room temperature after adding 27 N-sulphuric acid, the bromine water and vanadate are then added separately and the tubes heated in a water bath at 50° for 20 min. In the modification suggested here the bromide-bromate-vanadate mixture is added immediately after the sulphuric acid, when the temperature reaches a maximum of approx. 50°. A slightly higher yield of pentabromoacetone is obtained if the reaction is allowed to take place at room temperature, as Goldberg & Bernheim (1944) have shown, but this advantage is outweighed by the delay involved in cooling the tubes after the addition of the sulphuric acid.

METHOD

Reagents

Sulphuric acid. 27 N-H_2SO_4 was prepared by diluting 750 ml. of the pure concentrated acid to 1 l.

Bromide-bromate-vanadate solution. This contained 19·836 g. KBr, 5·440 g. $KBrO_3$ and 12·000 g. NH_4VO_3 in 1 l. This solution is stable for at least 3 months.

Ferrous sulphate solution. 22·0% (w/v) $FeSO_4.7H_2O$ (A.R.) in N-H_2SO_4. This solution is stored under hydrogen.

Sodium sulphide (A.R.). 2% solution freshly prepared.

Sodium sulphate. Anhydrous.

Light petroleum, b.p. 80–100°. A.R. aromatic-free grade was found satisfactory.

Citric acid stock solution. 0·2187 g. of the crystalline acid ($C_6H_8O_7.H_2O$)/l. in N-H_2SO_4. 25 ml. of this solution diluted to 250 ml. with N-H_2SO_4 contain 100 μg. anhydrous citric acid/5 ml.

Procedure

5 ml. 27 N-H_2SO_4 are added to 5 ml. of the citric acid solution contained in a test tube (150 × 24 mm.), fitted with a ground-glass stopper and, after mixing, 5 ml. of the bromide-bromate-vanadate reagent are run in from a pipette. The tube is stoppered, the contents mixed and allowed to stand for 20–30 min. Ferrous sulphate solution (2 ml.) is then added from a burette connected to a storage bottle and, after mixing, the tube is allowed to stand for 10 min. with occasional shaking to facilitate the removal of Br_2 from the air space. When all the Br_2 has been destroyed the solution is emerald green in colour, probably due to the formation of the hypovanadate ion, $V_2O_6^{4-}$. Light petroleum (6 ml.) is added and the tube is shaken for 1 min. either mechanically or by hand. The lower aqueous layer is then sucked off as completely as possible by means of a 20 or 25 ml. fine-tipped pipette fitted with a rubber extension tube for convenient suction. Sufficient anhydrous Na_2SO_4 is added to absorb the remaining drops of acid and prevent them from running out, and the petroleum is poured into a dry test tube. A 5 ml. portion is pipetted into a dry stoppered tube (125 × 18 mm.) and shaken by hand for 1 min. with Na_2S solution; the volume of this solution used was usually 3 ml. for a Spekker absorptiometer, and 4 ml. for a Unicam spectrophotometer. The yellow aqueous layer is sucked off with a dry pipette and filtered on a dry paper (Whatman no. 1, 4·25 cm.) into a test tube or colorimeter tube.

The absorption of the solution is measured with a Spekker absorptiometer using a 2 cm. microcell and Ilford no. 601 (spectrum violet) filters, or with a Unicam spectrophotometer set at 450 mμ., using an 0·5 in. tube. If the latter is used the colours are developed three or four at a time and the colour intensification of each solution is followed by taking readings of each in turn until steady maxima are obtained. With a Spekker it is more convenient to measure the light absorption of a batch of eight to ten solutions from 15 to 30 min. after colour development. Colour fading is not serious over this period in subdued light.

If the original solutions contain very small amounts of citric acid 10 ml. may be taken, and double quantities of H_2SO_4, bromide reagent and $FeSO_4$ added; 6 ml. of petroleum are still used for extraction. Tubes 150 × 28 mm. are a suitable size.

With these modifications it is possible to complete up to thirty determinations (including standards) in a normal working day.

Precautions. Care must be taken to avoid the two main sources of error stressed by Weil-Malherbe & Bone (1949), namely the incomplete reduction of free Br_2 and the contamination of the petroleum extract with acid. Incomplete removal of Br_2 may be caused either by deterioration of the $FeSO_4$ during storage or by inadequate shaking, and is easily detected by smelling before the addition of the petroleum. Acid contamination should not occur if sufficient Na_2SO_4 is added and if all glassware with which the Na_2S comes in contact is scrupulously clean.

Colour stability. The yellow colour is somewhat unstable. Initially its intensity increases gradually, reaching a maximum 12–15 min. after the end of the shaking period. Thereafter it slowly fades, particularly if exposed to strong light. In their discussion of colour stability Weil-Malherbe & Bone emphasize the importance of temperature on the intensity of the yellow colour and this has been confirmed. Hence a calibration curve should be prepared with each batch of determinations.

Accuracy. The calibration curve with 20–200 μg. citric acid is a straight line, but it seldom runs exactly through the origin. Data showing the degree of accuracy of the method compared with the results reported by Weil-Malherbe & Bone are given in Table 1. Recoveries of known quantities of citric acid added to different amounts of a dilute HCl extract of bone, the citric acid content of which was simultaneously estimated, were studied. The results of a particular experiment are given in Table 2.

Table 1. *Analysis of pure citric acid solutions*

(Using a Unicam spectrophotometer set at 450 mμ. with 0·5 in. tubes and 4 ml. Na_2S solution for colour development.)

Anhydrous citric acid (μg.)	No. of determinations	Coefficient of variation
20	15	3·51
50	15	1·64
100	12	1·87 (4·14)*
200	14	1·28 (1·57)*

* Corresponding figures calculated from the data presented by Weil-Malherbe & Bone (1949).

Table 2. *Recoveries of pure citric acid added to dilute hydrochloric acid extracts of bone*

	Citric acid (μg.)			
Bone extract	Added	Total found	Recovery	Recovery (%)
58·3	12·0	70·6	12·3	102·5
58·3	24·0	82·0	23·7	98·8
58·3	48·0	106·4	48·1	100·2
87·5	16·0	103·0	15·5	96·9

SUMMARY

A modified procedure for the estimation of 20–200 μg. of citric acid is described which is well adapted to the routine examination of large numbers of samples.

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The Chemical Assay of Progesterone

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The amounts of circulating progesterone in human blood have been deduced from the content of pregnane-3α:20α-diol in urine, but this method is open to criticism on the ground that no correlation has been proved between the urinary excretion of pregnanediol and the level of the circulating progesterone. Further, pregnanediol may not be a metabolite only of progesterone, nor its only metabolite. Deoxycorticosterone, pregn-5-en-3β-ol-20-one and cholesterol are also precursors of pregnanediol, and a number of related C_{21} steroids have been isolated from urine (Pearlman, 1948). Under pathological conditions, other precursors of pregnanediol and metabolites of progesterone might assume greater relative importance and invalidate an assumption perhaps adequate under physiological conditions. In the domestic animals, the identity of the urinary metabolites of progesterone is uncertain.

A direct method of assay should overcome these difficulties, but the development of a technique of sufficient sensitivity to determine the low concentrations of progesterone present in body fluids has proved difficult. The methods that have been used for the assay of progesterone may be divided into biological and chemical. Emmens (1950) has reviewed the biological methods.

A chemical approach to the problem of progesterone assay was made by Reynolds & Ginsburg (1942) who developed a quantitative method based on the ultraviolet absorption at 240 mμ. of Δ^4-3-ketosteroids like progesterone. Haskins (1950) considered that the relatively high extinction coefficient at 240 mμ. made progesterone readily measurable at optimum concentrations of 1–20 μg./ml. of ethanol.

Butt, Morris, Morris & Williams (1951) developed a method comprising extraction and partition between organic solvents, final separation of the individual steroids by chromatographic partition between aqueous methanol and *n*-hexane on Supercel columns, and subsequent polarographic estimation as a 3:20-diketosteroid. Recovery of progesterone added to plasma varied from 85 to 96 %, and concentrations of 0·1 μg./ml. of blood could be detected. The chromatographic separation of the individual steroids overcame the possibility of interference by other substances in the subsequent estimation, as was the case with the technique of Haskins (1950).

The present paper describes a method of assay based on extraction and purification of the hormone by partition between organic solvents (Butt *et al.* 1951), final separation by chromatographic partition on filter paper (Bush, 1952), and subsequent estimation by ultraviolet-absorption spectroscopy (Reynolds & Ginsburg, 1942).

EXPERIMENTAL METHODS

(A) *Extraction of biological material and preliminary fractionation*

Two methods were investigated for the extraction and preliminary fractionation of progesterone from body fluids, necessary before its isolation by chromatographic technique may be attempted. The method first investigated differed from the final, second method described below in minor details and in employing $CHCl_3$ instead of ethyl acetate.

The second method was adapted from that of Butt *et al.* (1951) and is illustrated by the following example: oxalated blood (40 ml.) was added with stirring to 100 ml. of a 3:1 (v/v) ethanol-ethyl ether mixture. The mixture was stirred for 5 min., centrifuged, the supernatant decanted and the precipitate washed twice by stirring with 50 ml. portions of the 3:1 ethanol-ethyl ether mixture, and separated by centrifuging. The combined extracts were concentrated *in vacuo* to about 20 ml. and diluted with 40 ml. distilled water. The diluted solution was extracted with 3 × 60 ml. ethyl acetate and the combined extracts evaporated to dryness *in vacuo*. The residue was transferred to a centrifuge tube by washing the flask three times with 3 ml. portions hot 70 % aqueous methanol. The mixture was stored for 18 hr. at −15°, and then centrifuged at −10°. The supernatant was decanted, diluted with 20 ml. distilled water, and extracted with 3 × 30 ml. light petroleum (b.p. 40–60°). The combined extracts were washed with 2 × 30 ml. water, reduced *in vacuo* to about 1 ml. and transferred with two washings of 0·5 ml. portions of light petroleum to a glass evaporating dish, for evaporation to dryness *in vacuo* over $CaCl_2$. When dry, the residue was dissolved in about 0·1 ml. benzene and transferred to the chromatogram filter paper with two further washings of 0·1 ml. portions of benzene.

(B) *Final separation of progesterone by chromatographic methods*

Several chromatographic systems were investigated, the first two following the techniques described by Bush (1950, 1951*a*, *b*, 1952), which were evolved for the study of adrenocortical steroids, but which are applicable to progesterone.

(i) *Alumina paper*

Preparation of papers was carried out as described by Bush (1952). Papers of optimum adsorption were found to be rough in surface, to have a pH of about 7·5 as measured by B.D.H. Universal indicator, and when a portion was dipped in a saturated solution of iodine in light petroleum (b.p. 60–80°), it became, on drying, a yellow-brown colour.

The chromatography was carried out by the ascending technique in a glass jar measuring 11 × 4 × 15 in. high, the alumina paper dipping into mixtures of benzene and $CHCl_3$ in proportions which varied according to the absorptive qualities of the paper. It was found necessary to run trial chromatograms to determine the optimum proportions of the two solvents for each preparation of papers. With a paper of optimum absorption activity a mixture of 3 parts of benzene to 1 of $CHCl_3$ gave a good separation of the steroids tested.

Detection of spots was done in two ways. (*a*) After drying at room temperature the chromatogram was dipped into a saturated solution of iodine in light petroleum, and the steroid spots demonstrated and recorded by photography on Ilford Reflex Contact paper no. 50, in ultraviolet light from a lamp whose major output was in the region 365 mμ.

(*b*) The dried chromatogram without immersion in iodine solution may also be photographed on the contact paper, in ultraviolet light from a lamp (ViTan Mercury Vapour Burner, Thermal Syndicate Ltd., London, S.W. 1) with maximum output in the region 254 mμ. This method is preferable as the steroid may be recovered from the paper, free from iodine.

(ii) *Partition on filter paper*

(*a*) *Light petroleum-80% aqueous methanol.* The system follows that described by Bush (1952). Two glass tanks, 11·5 × 7 × 20·5 in. high, were enclosed in an insulated cabinet with inside measurements of 36 × 22 × 30 in. high. Heat was supplied by four asbestos woven resistance nets 6·5 × 8 in., resistance 150 Ω (Cressal Manufacturing Co., Birmingham 19). Two of these were in series at one end of the cabinet, shielded by an asbestos baffle plate, and were in parallel with two other nets similarly arranged at the other end of the cabinet, which gave a total wattage of approximately 267. The temperature was controlled by a thermostat (Sunvic T.S. 1, Sunvic Controls Ltd., London, W.C. 2). The air was circulated by a fan, 14 in. diam., suspended from the lid, with the motor outside. This gave a maximum spatial difference of about 1°. The temperature was maintained at 34°. The arrangement of the tanks allowed the running of four chromatograms at one time, two in each tank.

Two wads of filter paper were suspended in the tank, against the longer walls, one soaking up the mobile phase (light petroleum b.p. 80–100°), which covered the bottom of the tank to a depth of about 2 cm., and the other the stationary phase (80% aqueous methanol), which was contained in a trough holding 50–100 ml. The tank was sealed by Plasticine pressed round the outside of the junction between the walls and the lid.

Whatman no. 1 filter paper was cut into rectangles 20 cm. wide and 50 cm. long. The start line was ruled 6 cm. from one end of the paper, and a 2 cm. length dipped into an upper trough containing the mobile phase, being supported by the weight of a glass rod passed through two slits cut 1 cm. from the end of the paper. A descending flow of 38 cm. took about 120 min., the flow being faster the further the paper was hung from the aqueous methanol-soaked pad and the nearer the light petroleum pad. It was essential to hang the chromatogram paper parallel to the solvent pads, and not at an angle, to avoid an oblique solvent front.

Improved chromatograms were obtained by chromatographically washing the paper with light petroleum and drying at room temperature before use. This removed from the paper some of the impurities which absorbed ultraviolet light.

The extracts in about 0·3 ml. of benzene were added to the paper as two parallel lines about 5 mm. apart, at right angles to and reaching the previously selected start line. The extracts were then concentrated by running an ascending flow to the start line with a mixture of ethyl acetate, $CHCl_3$ and methanol (5:5:1; v/v), in which mixture progesterone had R_F 1·0.

After concentrating the extracts at the starting line, the paper was suspended in the tank and left overnight to equilibrate. At the same time the mobile phase was left in the cabinet to attain the temperature of 34°, and the next day was poured into the upper trough through a hole in the glass lid by means of a funnel. The hole was resealed immediately to prevent any change in the equilibrium within the tank. The light petroleum on the bottom of the tank was replenished after each run by adding the solvent remaining in the upper trough, and the 80% aqueous methanol in the lower trough was replaced as often as necessary. It is usual for the two phases in any system to be mixed and allowed to separate at the operating temperature, but as these two phases are almost completely immiscible, good chromatography was achieved without prior mixing.

The chromatogram was dried in air at room temperature and pinned down over Ilford Reflex Contact paper no. 50, better contact being obtained on a convex surface formed by bending a sheet of three-ply wood (Markham & Smith, 1949). The chromatogram was then exposed for 2 sec. to ultraviolet light, at 60 cm. from a lamp emitting most of its energy in the 254 mμ. region.

(*b*) *Light petroleum-water-cellulose complex.* It was clearly desirable to establish a second chromatographic system in which to examine the behaviour of substances separated by the method just described, and in which progesterone could, if possible, be made to run with a lower R_P. Consistency of behaviour in a second system constitutes a more definite identification of an unknown substance. The apparatus and methods described for the light petroleum-80% aqueous methanol system were used with the other systems investigated, modifications being mentioned as necessary.

Chromatograms of pure progesterone and of extracts of blood to which progesterone had been added were run with light petroleum (b.p. 80–100°) on Whatman no. 1 filter paper. Using the descending flow system, equilibration times of 18 and 48 hr. and temperatures of 18 and 34° were employed.

(*c*) *Aqueous propanol-water-cellulose complex.* Various proportions of propanol in distilled water were used to run, on Whatman no. 1 filter paper, chromatograms with pure progesterone and with extracts of blood to which progesterone had been added. Equilibration lasted for 16–18 hr. and the chromatograms were run with the descending system at 18°.

(iii) *Reversed phase chromatography*

Kritchevsky & Tiselius (1951) described the reversed phase partition chromatography of steroids on silicone-treated paper, in which the non-polar phase is held stationary and the more polar phase is the moving one. The following two experiments were based on their technique.

Preparation of paper. A sheet of Whatman no. 1 filter paper, 10 × 50 cm., was drawn through a 5 % (v/v) solution of Dow Corning Silicone no. 1107 in CCl_4, blotted between absorbent paper and baked for 2 hr. in an oven at 96°.

(*a*) *Aqueous ethanol-chloroform.* This is the solvent system used by Kritchevsky & Tiselius. Water, absolute ethanol and $CHCl_3$ (6:10:10; v/v) were mixed and kept 1 hr. at room temperature. The upper phase which separated out was the more polar. The non-polar phase was run into the bottom of the glass chromatography jar, and the chromatogram developed with the more polar ethanol-water phase in the descending manner, after the paper had hung in the atmosphere of the stationary phase for 1 hr. After running, the chromatogram was dried for 15 min. at 96°, and the spots detected by exposing to ultraviolet light over contact paper. Various proportions of the solvents were examined, with and without prior mixing of the two phases.

(*b*) *Aqueous methanol-benzene.* Silicone-treated paper, prepared as previously described, was used with benzene as the stationary phase and various proportions of water and methanol as the mobile phase. The experiments were carried out at room temperature, both with and without prior mixing and separation of the two phases, and at 34° with prior mixing of the phases.

(C) *Elution of progesterone from the chromatogram*

The position of the spot of progesterone on the chromatogram was determined by placing the contact photograph behind and in contact with the paper, positioning by means of the pin holes made at the time of photographing. A source of light behind the contact photograph showed the spots through the filter paper, and these were outlined lightly in pencil. A piece of paper, slightly larger than the area of the spot, was cut out and tapered to a point at one end, and another piece of the same size was cut from an area at the same level on the chromatogram, to be used after elution as the blank solution in the subsequent ultraviolet-absorption analysis. The apparatus for elution consisted of a reservoir above a bent capillary tube, with a stainless steel wire for controlling the flow of eluent, and a small plate of glass held by a rubber band to press the paper against the lower end of the capillary. A short length of fine glass rod, bent double, guides the eluate from the lower end of the paper into the receiving tube. The apparatus was suspended through a hole in the lid of a glass jar. Between 2 and 3 ml. 96 % ethanol were used as eluent, and the atmosphere was saturated with the vapour from this solvent by pouring a few ml. on to the floor of the jar.

(D) *Estimation of progesterone by ultraviolet absorption spectroscopy*

Amounts of crystalline progesterone varying from 60 to 3·75 μg., eluted in 3 ml. 96 % ethanol, were examined with ultraviolet light in a Beckman Model D.U. spectrophotometer against equivalent blanks from the same level on the chromatogram. Fused silica cells 1 cm. wide and with a capacity of 2·5 ml. were used. The extinction coefficient, *E*, for each concentration was measured from 224 to 276 mμ. and, from quantitative comparisons made at the absorption maximum at 240 mμ., characteristic of the Δ^4-3-ketosteroids (Reynolds & Ginsburg, 1942), ϵ_{240} was calculated.

To demonstrate the possibility of interference in the spectroscopy by impurities from the filter paper, the absorption spectrum of the eluted blank was compared with 96 % ethanol.

(E) *Recovery of progesterone added to blood* in vitro

To 40 ml. samples of oxalated blood from the external jugular vein of a castrated male sheep were added varying amounts of crystalline progesterone in solution in small volumes of 96 % ethanol. After shaking for 30 sec., extraction, chromatographic partition and spectroscopic estimation were carried out as previously described. The same volume of blood, without the addition of progesterone, was similarly examined as a control. To discover any effect on progesterone of blood *in vitro*, a sample was incubated for 1 hr. at 37°, after the addition of progesterone and before the commencement of extraction.

RESULTS

(A) *Extraction of biological material and preliminary fractionation*

Using the first method, together with the alumina-paper chromatographic system described, recoveries of progesterone added to blood *in vitro* were of the order of 50 % as judged by the size and density of the chromatogram spots obtained, compared with spots containing known amounts of pure progesterone.

Table 1. *Recovery of progesterone added to 40 ml. blood, using second method of fractionation and paper chromatography with light petroleum-80 % aqueous methanol*

Progesterone added			Progesterone recovered		
(μg.)	In solution in 96 % ethanol (μg./ml.)	*E*	*E*	(μg./ml.)	(%)
60	20	1·100	0·870	15·8	79
45	15	0·820	0·632	11·6	77
30	10	0·550	0·392	7·2	72
15	5	0·275	0·205	3·7	74
4	1·3	0·070	0·055	0·9 ·	69
		After incubation for 1 hr. at 37°			
30	10	0·550	0·415	7·6	76

The second method combined with the light petroleum-80% aqueous methanol chromatographic system described, gave recoveries of the order of 75% of progesterone added to blood (Table 1).

(B) *Final separation of progesterone by chromatographic methods*

(i) *Alumina paper*

Quantities of pure progesterone from 2 to 50 μg. could be detected, but the R_F values varied slightly with the amounts of the steroid present, tending to be greater for larger amounts. Good separations were obtained of progesterone (R_F 0·75), oestrone (R_F 0·46), oestradiol (R_F 0·21) and oestriol (left on origin), with a mixture of benzene and chloroform (3:1; %) run on a paper of optimum absorption activity. Several estimations were made of known amounts of progesterone added to blood, recoveries being of the order of 50%. Incubation of the blood for 1 hr. at 37°, after the addition of progesterone and before the commencement of the extraction process, did not reduce the recovery rate.

(ii) *Partition on filter paper*

(*a*) *Light petroleum-80% aqueous methanol.* A mixture of progesterone, 11-deoxycorticosterone 21-acetate, and testosterone was efficiently separated into its components, having, respectively, R_F values of 0·85, 0·70 and 0·40. Amounts of crystalline progesterone, ranging from 1 to 60 μg., were handled quantitatively by the system, no variation in R_F occurring due to concentration of the material.

(*b*) *Light petroleum-water-cellulose complex.* At room temperature pure progesterone ran with an R_F of about 0·50, but there was considerable 'trailing' and larger amounts had a higher R_F. The results were the same with equilibration times of 18 or 48 hr. At 34°, the R_F was slightly increased but the variability with the amount of steroid remained. Reasonable recovery was obtained of 20 μg. of progesterone added to 10 ml. plasma, after extraction and running the chromatogram at 34°, but there was considerable 'trailing', and some progesterone remained on the starting line.

(*c*) *Aqueous propanol-water-cellulose complex.* 10% Aqueous propanol gave a very diffuse spot of pure progesterone at R_F 0·42. A compact spot at R_F 0·90 was obtained with 25% aqueous propanol, while 15% gave fairly compact spots at R_F 0·69, and handled quantitatively a series of from 1 to 16 μg. of pure progesterone. Extracts of blood to which progesterone had been added were examined with 15% aqueous propanol, but progesterone remained at the starting line along with most of the extracted impurities, although the control crystalline progesterone gave the expected chromatographic response.

(iii) *Reversed phase chromatography*

(*a*) *Aqueous ethanol-chloroform.* When chloroform was mixed with and allowed to separate from various proportions of absolute ethanol and water, the R_F of progesterone increased with the increase of the proportion of absolute ethanol, values for 50, 60 and 66% ethanol being, respectively, 0·07, 0·09 and 0·13. Testosterone with 60% ethanol had R_F 0·51.

When extracts of blood to which progesterone had been added were run chromatographically in these systems, the progesterone was held at the starting line with the impurities from the blood extract. When chloroform was the stationary phase, and various proportions of absolute ethanol and water the mobile phase, without prior mixing with the chloroform, compact spots of pure progesterone were obtained, the values for 50, 60 and 95% ethanol being, respectively, 0·39, 0·80 and 0·96. Again, however, progesterone was held at the starting line by impurities in the blood extracts run in these systems at 18°. When pure progesterone was added at the starting line after and on top of such an extract, it moved with only a slightly lower R_F than the control pure hormone.

(*b*) *Aqueous methanol-benzene.* When the mobile and stationary phases were mixed and allowed to separate, and the chromatograms run at room temperature or at 34°, in every case the chromatography was a failure due to the removal of silicone from the paper by the benzene in the mobile phase. In the same way it was found that when substances were concentrated at the starting line by the preliminary run with the ethyl acetate-chloroform-methanol mixture, there was partial removal of silicone from the paper, although this did not appear to interfere with the subsequent chromatography. When the phases were not mixed, compact spots with the R_F values shown in Table 2 were obtained at 18°.

Table 2. *Reversed phase chromatography with varying proportions of methanol and water without prior mixing with benzene*

Stationary phase	Mobile phase. Aqueous methanol	R_F Progesterone	R_F Testosterone
Benzene	50%	0·10	0·50
Benzene	60%	0·35	0·81
Benzene	70%	0·68	0·90

When an extract of progesterone-treated blood was run chromatographically in the 60% aqueous methanol-benzene system, the recovered progesterone ran with an R_F of 0·35 alongside the pure substance, although there was a 'trial' to the starting line. Running the chromatogram with 70% aqueous methanol gave compact spots of both

progesterone and testosterone, and gave a complete separation of the former steroid from extracted impurities.

(C) *Elution of progesterone from the chromatogram*

Complete recovery of the spot of progesterone was obtained. This was confirmed by ultraviolet photography of the eluted piece of paper and by estimation by ultraviolet-absorption spectroscopy.

(D) *Estimation of progesterone by ultraviolet absorption spectroscopy*

The absorption spectra of three concentrations of pure progesterone in 96% ethanol are shown in Fig. 1. From the data in Table 3, E for a solution containing 1 μg./ml. at 240 mμ. $(xy)/x^2$ was found to be 0·056, and hence $\epsilon_{240} = 17{\cdot}6 \times 10^3$.

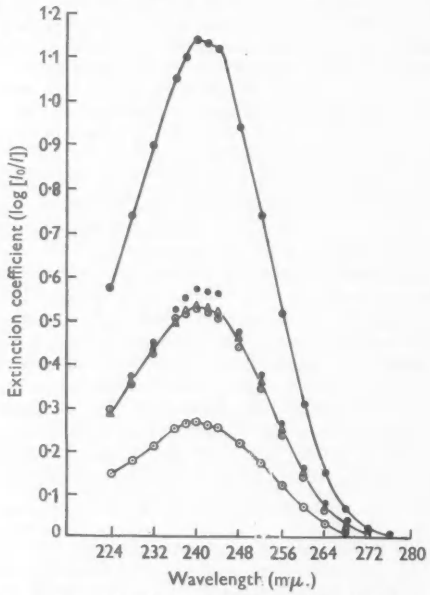

Fig. 1. Ultraviolet absorption spectra of three concentrations of pure progesterone. ●—●, 20 μg./ml.; △—△ 10 μg./ml.; ⊙—⊙, 5 μg./ml.

The extinction coefficients of the greatest and the least have also been adjusted to the intermediate concentration.

The filter paper was found to contain material which absorbed strongly in the same region as progesterone, and if the eluted blank portion of paper differed in size or position on the chromatogram from that containing the progesterone spot, the absorption spectrum of the latter was distorted, and the absorption maximum differed from 240 mμ. Prior chromatographic washing of the filter paper with light petroleum reduced this contamination.

(E) *Recovery of progesterone added to blood* in vitro

Recovery of progesterone added to blood *in vitro* was of the order of 75%, and no further loss resulted after incubation at 37° for 1 hr. (Table 1). On no occasion was progesterone found in oxalated blood from a castrated male sheep when the hormone had not been previously added, which means that less than 4 μg. were present in 40 ml. blood.

Table 3. *The application of the method of 'least squares' to the extinction coefficient of various concentrations of progesterone at* 240 mμ.

Concentration of pure progesterone (x) (μg./ml.)	Extinction coefficient at 240 mμ. (y)	xy	x^2
20	1·140	22·80	400
15	0·840	12·60	225
10	0·530	5·30	100
5	0·262	1·31	25
2·5	0·139	0·35	6·25
1·25	0·055	0·07	1·56
		42·43	757·81

DISCUSSION

Recoveries of the order obtained by the second extraction method were considered reasonable in view of the number of steps in the procedure, and this has been adopted as the first stage of the assay technique.

Some difficulty was found in preparing alumina papers of standard quality, considerable variation occurring between one batch and the next. The elimination of the displacement effects due to impurities would demand tedious prior purification of biological extracts. In view of the disadvantages found, this chromatographic method was abandoned.

In the light petroleum-80% aqueous methanol chromatographic system ((ii) (*a*)), progesterone ran at a high R_F, and there was a tendency for incomplete separation from impurities travelling at or near the solvent front. In such a case, complete separation was usually achieved by eluting and rerunning the material in the region of R_F 0·85. The technique gave an efficient separation and semiquantitative estimation of progesterone and has been adopted as the second stage of the assay method.

It appears that the reversed phase partition system devised by Kritchevsky & Tiselius (1951) achieves an effective separation of pure substances, but is unable to separate these from the impurities contained in blood extracts. The properties of the silicone-treated paper were apparently changed by washing with organic solvents. The reversed phase system evolved ((iii) (*b*)) has proved valuable in the examination of naturally occurring progesterone

found in extracts of biological material by the use of the partition system ((ii) (*a*)).

Progesterone in solution in 96% ethanol is accurately and conveniently estimated by ultraviolet absorption analysis in a Beckman spectrophotometer, and this constitutes the third stage of the assay method. The results indicate a close adherence to Beer's Law, and in this respect differ from those of Reynolds & Ginsburg (1942) who found no proportional relationships between the extinction coefficients of different concentrations of progesterone, and also that the spectrum varied at different concentrations. The conclusion of Haskins (1950) is confirmed, that the hormone is readily measurable at concentrations of 1–20 μg./ml. ethanol.

The finding that blood, at 37° for 1 hr., has no effect on progesterone conflicts with the statement by Hooker & Forbes (1949) that progesterone disappears rapidly from plasma stored at body temperature, and suggests that it is not progesterone which is assayed by their method. It indicates also that the rapid disappearance from the circulation of intravenously injected progesterone reported by Haskins (1950) and Butt *et al.* (1951), is due to utilization by the tissues and not to constituents of the blood. Hooker & Forbes (1947) found 0·33–8·25 μg. progesterone per ml. blood of ovariectomized mice, 6–7 hr. after the subcutaneous injection of 2 mg. of the hormone in oil, but Haskins (1950) was unable to demonstrate its presence over a period of 3 days in the blood of a rabbit injected intramuscularly with 50 mg. in oil. The interval after the injection when the first blood sample was taken is not reported.

Examples of the application of the technique to biological material have been submitted for publication elsewhere (Edgar, 1952).

SUMMARY

1. The development of a chemical technique for the detection and determination of progesterone in biological material is described. It is based on the extraction of the hormone and its partition between organic solvents, its final separation by chromatographic partition on filter paper and its subsequent estimation by ultraviolet absorption spectroscopy.

2. Work on the chromatography of steroids has resulted in the development of a reversed phase system capable of separating progesterone from impurities, which is also of value in establishing the identity of substances isolated from biological material.

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The Preparation and Properties of β-Glucuronidase

6. ACTIVITY IN RAT-LIVER PREPARATIONS

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Previous papers in this series have dealt with the fractionation and activity of β-glucuronidase in mouse-liver suspensions ('homogenates'). Identical fractionation of the enzyme was achieved by centrifuging at high speeds or by buffering to slightly acid pH. The sedimentable fraction was associated with subcellular particles of all sizes, and these were agglutinated in acid buffers. In water suspensions (Kerr & Levvy, 1951; Walker & Levvy, 1951), the sedimentable fraction was fully active at the usual tissue concentrations employed for β-glucuronidase assay with phenolphthalein glucuronide, and accounted for slightly less than half the total enzyme activity in preparations of normal adult liver.

In suspensions of mouse liver prepared in isotonic media (Walker, 1952), nearly all the enzyme was in the sedimentable fraction. These preparations, however, did not display full glucuronidase activity until some measure of disruption of the subcellular particles had occurred, with release of part of their enzyme content to the solution. This process of activation was irreversible, and was considered to be due to readier access of the substrate to the enzyme remaining in the particles. Soluble enzyme in mouse-liver suspensions was fully active under all conditions of assay. In this connexion, soluble enzyme was defined as that fraction which was not sedimented after 15 min. at 25000 *g* nor precipitated by acetate buffer, and which gave optically clear preparations under the phase-contrast microscope (Walker & Levvy, 1951).

A similar approach has been made to the study of β-glucuronidase in rat liver, in which part of the enzyme is known to be precipitable at acid pH (Becker & Friedenwald, 1949). A complication was encountered at an early stage due to reversible inhibition of soluble and insoluble enzyme by material present in the tissue.

EXPERIMENTAL

Preparation and fractionation of the enzyme. In most of the experiments, hooded Lister strain rats were used. For purposes of comparison, a few later experiments were done with albino Wistar strain rats. The liver was ground for 1 min. at room temperature in the glass homogenizer with water or 0·25 M-sucrose solution, diluted with the homogenizing fluid to give a tissue concentration of 0·5–1 % (w/v), and sampled for the measurement of enzyme activity. As described for mouse liver (Walker & Levvy, 1951), other samples were fractionated at room temperature, either by adding acetate or citrate buffer (pH 5·2, final concentration 0·1 N) and centrifuging at low speed (1500 *g* for 15 min.), or by centrifuging the unbuffered preparation at high speed (25000 *g* for 15 min.). With buffered preparations, both supernatant and sediment were taken for assay, the latter being washed and resuspended in the buffered homogenizing fluid with the aid of the glass homogenizer. After centrifuging unbuffered preparations at high speed, only the supernatant could be sampled for assay since the sediment was not well enough packed to permit complete separation of the two fractions.

Enzyme assay. The enzyme preparations, fractionated and unfractionated, were diluted if necessary to a final volume of 200 ml./g. moist liver, and 0·5 ml. was transferred to a tube containing 0·5 ml. 0·01 M-phenolphthalein glucuronide solution (Talalay, Fishman & Huggins, 1946) and 3 ml. 0·1 N-acetate or citrate buffer, as a rule pH 5·2. Where buffer had been used in the fractionation, the same buffer was used in the assay. The remainder of the procedure followed that described by Kerr & Levvy (1951). Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μg. phenolphthalein in 1 hr. at 37°.

Under the above conditions of assay, the enzyme in preparations of rat liver did not always display full potential activity (see below). The latter could be most conveniently measured by adding the surface-active agent, Triton X-100 (Rohm and Haas Co.), as a solution in the buffer, to the incubation mixture to give a final concentration of 0·075 % (w/v).

Endogenous inhibitor. Preparations of the inhibitor for rat-liver glucuronidase present in this and other tissues (see below) were obtained free from enzyme activity by immersing a water suspension of the tissue in a boiling-water bath for 2 min. The cooled preparation was made 0·1 N with respect to acetate or citrate buffer, pH 5·2, and unless stated to the contrary was diluted to give a final tissue concentration of 0·5 % (w/v). To measure inhibitory power, 0·5 ml. of the preparation was added in place of buffer to the incubation mixture during the assay of preparations of soluble, inhibitor-free enzyme. The order of addition of enzyme, inhibitor and substrate did not influence the measure of inhibition. Very often, inhibitor and enzyme were prepared from separate samples of the same liver preparation.

RESULTS

The action of an endogenous inhibitor in water suspensions of rat liver

The following series of experiments, based on those previously done with mouse liver (Kerr & Levvy, 1951; Walker & Levvy, 1951), led to the belief that glucuronidase in water suspensions of rat liver is subject to the action of an endogenous inhibitor.

Kerr & Levvy (1951) found that the use of too high a tissue concentration in the assay of water suspensions of mouse liver led to anomalous results for glucuronidase activity. A similar phenomenon has been noted in dog and rat tissues by Fishman (1952). This effect was not seen in mouse-liver preparations (Kerr & Levvy, 1951) at the usual tissue concentrations (about 2·5 %, w/v) taken for assay by the phenolphthalein glucuronide method, nor at any tissue concentration once all the enzyme in the preparation had been converted into a soluble form. The effect of varying the tissue concentration on the glucuronidase activity of acetate-buffered water suspensions of rat liver is shown in Table 1. Addition

Table 1. *Effect of tissue concentration on the glucuronidase activity of water suspensions of rat liver (hooded Lister strain)*

(Serially diluted suspensions assayed in acetate buffer, pH 5·2, in presence and absence of 0·075 % Triton X-100. Results expressed as G.U./mg. moist liver. For definition of G.U. in tables see text.)

Tissue concentration* (g. liver/100 ml. suspension)	G.U./mg. liver: Normal adult liver, With Triton	Normal adult liver, Without Triton	Infant (4-day-old) liver, With Triton	Infant (4-day-old) liver, Without Triton
1·0	27·6	16·1	15·8	4·8
0·5	26·8	15·6	14·8	4·8
0·25	26·4	18·4	14·8	5·4
0·13	26·4	21·0	15·0	7·6
0·06	27·0	22·5	14·2	8·5

* These concentrations reduced to one-eighth in the assay tubes.

of Triton X-100 was adopted as a convenient way of making all the enzyme soluble, as found for mouse liver by Walker & Levvy (1951). A 0·5 % suspension of rat liver had the same order of glucuronidase activity as a 2·5 % suspension of mouse liver, but even at this or greater dilution rat-liver preparations in absence of Triton X-100 still varied in enzyme activity according to the tissue concentration employed, and never attained the constant values observed in presence of the surface-active agent. The percentage differences were greater with infant than with adult liver. A tissue concentration of 0·06 % (w/v) was the lowest giving measurable release of phenolphthalein under the assay conditions. Triton X-100 was equally effective in concentrations in the assay tubes ranging from 0·015 to 0·375 % (w/v).

As previously found for mouse liver (Kerr & Levvy, 1951), the glucuronidase activity in water suspensions of rat liver could be fractionated on the low-speed centrifuge after buffering to pH 5·2 with acetate or citrate. Fig. 1 shows results thus obtained, the activities before and after fractionation being measured in presence and absence of Triton X-100. A single adult- or infant-liver preparation was used for all measurements, and fractionation was done immediately or after 2 hr. incubation in the buffer. Incubation in acetate or citrate is a preliminary to the preparation of tissue extracts for some methods of glucuronidase assay.

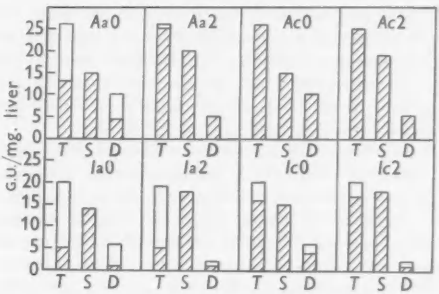

Fig. 1. The glucuronidase activity in presence and absence of 0·075 % Triton X-100 of water suspensions of rat liver (hooded Lister strain), buffered with acetate or citrate to pH 5·2 and fractionated before and after incubation at 37° for 2 hr. All preparations were diluted to 200 ml./g. liver before assay. Results are expressed as G.U./mg. liver. For definition of G.U., see text. The total height shows the activity in presence of Triton X-100, and the height of the hatched area the activity in its absence. *T*, unfractionated suspension; *S*, soluble fraction; *D*, sedimentable fraction; *A*, adult liver; *I*, infant (4-day-old) liver; *a*, buffered with acetate; *c*, buffered with citrate; *O*, fresh suspension; 2, incubated suspension.

Assays conducted in presence of Triton X-100 revealed a simple transfer of enzyme activity from the sedimentable to the soluble fraction during the incubation of the preparation in either buffer. There was no change in the activity of the unfractionated preparation which was always equal to the sum of the activities in the two fractions. Citrate and acetate gave similar fractionation of activity. (Control experiments showed that addition of Triton X-100 to the fresh preparation before fractionation made all the enzyme soluble.)

Assays in absence of Triton X-100 were more difficult to interpret. Considering acetate-buffered preparations first, the soluble fraction invariably displayed full enzyme activity, but the activity of

the sedimentable fraction separated prior to incubation was greatly depressed in absence of the surface-active agent. This was also true of the unfractionated suspension, so much so that it was exceeded in activity by the soluble fraction alone. After incubating the suspension for 2 hr. in acetate buffer, a difference became apparent between adult- and infant-liver preparations. The adult-liver preparation approached full activity, as did its sedimentable enzyme, whilst the infant-liver preparation retained its original depressed value, in spite of the fact that nearly all the enzyme had gone into solution. The disparity between fractionated and unfractionated preparations of infant liver assayed in absence of Triton X-100 thus became greater after incubation in acetate buffer. Although the inhibitor was lacking or without effect in separated soluble enzyme fractions, it must have acted on soluble enzyme in the unfractionated suspension. It is therefore evident that the effect of Triton X-100 in overcoming the endogenous inhibitor did not depend entirely on its action in converting all the enzyme in suspensions into a soluble form.

When citrate buffer was used instead of acetate, the effect of the inhibitor became smaller in infant-liver preparations and disappeared entirely in adult preparations. This was apparently due to citrate causing reversal of the inhibition since the use of other buffers (propionate, lactate, phthalate) or dilute hydrochloric acid to bring the pH to 5·2 gave results similar to those obtained with acetate.

Other series of experiments confirmed and extended the results shown in Fig. 1. The effect of fractionation on acetate-soluble enzyme was reversible: adding the sediment led to a net loss in activity. The inhibitor in infant (4-day-old) liver suspensions was stable to 6 hr. incubation in acetate buffer. Incubation for 2 hr. in acetate buffer reduced the effect of the inhibitor in liver from 30-day-old animals by about 50 % and, as we have already seen (Fig. 1), almost completely destroyed that in adult liver. ('Adult' rats were at least 3 months old.)

Immediate fractionation of acetate-buffered suspensions followed by incubation of the separate fractions for 2 hr. showed changes in the inhibitor, uncomplicated by changes in enzyme distribution. The activity of the inhibitor-free soluble fraction was unchanged by incubation, as was the total activity, measured in presence of Triton X-100, of the sedimented fraction. Incubation in acetate buffer destroyed the inhibitor in the sediment from adult liver, but left that from infant liver unaffected.

As proved later (Table 8), high-speed centrifuging (25000 *g* for 15 min.) of unbuffered water suspensions of rat liver led to fractionation of the total enzyme activity identical to that produced by acetate or citrate buffer, pH 5·2. The soluble fraction obtained on the high-speed centrifuge, however, contained some of the inhibitor, as shown in Table 2. Before incubation in acetate buffer, Triton X-100 caused a rise in activity in the soluble as well as the sedimentable fraction. Incubation in the buffer destroyed the inhibitor in both adult-liver fractions, but not in the infant-liver preparations.

Recentrifuging the high-speed supernatant at low speed after buffering with acetate freed the soluble enzyme from inhibitor which now sedimented. (This sediment always contained a trace of adsorbed enzyme which could not be removed by washing with the buffer.) The insolubility of the inhibitor in the buffer explains the fact that fractionation of buffered suspensions always yielded the soluble enzyme in an inhibitor-free state (cf. Fig. 1).

Table 2. *The glucuronidase activity in presence and absence of* 0·075 % *Triton X-100 of the soluble and sedimentable fractions separated by centrifuging unbuffered suspensions of rat liver at* 25000 *g for* 15 *min.*

(Hooded Lister strain rats were used. The fractions were buffered with acetate (pH 5·2, final concentration 0·1 N) and diluted to 200 ml./g. liver. Samples were taken for assay before and after 2 hr. incubation. One sample of the buffered high-speed supernatant, unincubated, was centrifuged at 1500 *g* for 15 min. and the sediment was discarded before assay. Results are expressed as G.U./mg. liver.)

	G.U./mg. liver				
	High-speed supernatant			Sediment*	
	With Triton	Without Triton	Acetate-soluble enzyme (without Triton)	With Triton	Without Triton
Adult male					
Before incubation	17·2	11·8	16·4	9·2	6·4
After incubation	17·4	17·4	—	9·2	8·8
Infant (5 days old)					
Before incubation	10·8	5·6	10·3	4·0	1·6
After incubation	10·8	6·4	—	3·8	1·4

* Total activities are low due to losses in the separation and washing of this fraction.

Table 3. *The effect of Triton X-100 (0·075 %, w/v) on the glucuronidase activity of water suspensions of various rat and mouse tissues*

(Rat preparations (hooded Lister strain) were diluted to 200 ml./g. moist tissue, and mouse preparations to 40 ml./g. Assays were done in acetate buffer, pH 5·2, and results are shown as the mean ± S.E.)

Tissue	Number of experiments	G.U./mg. moist tissue: With Triton	G.U./mg. moist tissue: Without Triton	Inhibition* (%)
Adult rat liver	7	25·94 ± 1·47	14·83 ± 1·86	43·9 ± 4·65
Infant rat liver	7	19·97 ± 1·57	4·40 ± 0·42	77·7 ± 2·02
Adult rat kidney	3	5·67 ± 0·09	3·93 ± 0·41	30·7 ± 5·25
Adult rat spleen	3	20·27 ± 0·87	19·07 ± 0·67	5·9 ± 0·09
Adult mouse liver	6	3·17 ± 0·15	3·10 ± 0·12	2·0 ± 0·63
Adult mouse kidney	5	3·20 ± 0·36	3·02 ± 0·28	5·8 ± 1·18

* The significance of the difference was tested by calculating t (= mean/S.E.).

For comparison with rat liver, a study was made of the effects of Triton X-100 on the glucuronidase activities of unfractionated water suspensions of rat kidney and spleen, and mouse liver and kidney (Table 3). Activation was marked in rat kidney and slight, but in every case significant ($P < 0·05$), in rat spleen and mouse liver and kidney. An occasional small increase in the activity of water suspensions of mouse liver on addition of Triton X-100 has previously been noted (Walker & Levvy, 1951).

Properties of the endogenous inhibitor. The following properties of the endogenous inhibitor in water suspensions of rat liver were confirmed in experiments with preparations of enzyme-free inhibitor (see Experimental section), tested against inhibitor-free, acetate-soluble enzyme. In the untreated preparation, it was partly insoluble and partly in solution, but it was completely precipitated, without loss in inhibitory power, by buffering to pH 5·2. Its action was completely reversed by Triton X-100 and in adult preparations by citrate also. The inhibitor from infant liver was stable to incubation in acetate buffer, pH 5·2, but the inhibitor from adult liver was destroyed after 2 hr. incubation. When precipitated from preparations of soluble enzyme by addition of acetate buffer, the inhibitor always adsorbed traces of enzyme which could not be removed by washing with the buffer.

Unbuffered suspensions of adult or infant liver could be boiled for at least 10 min. without loss in inhibitory power, and this was made the basis of the preparation of enzyme-free inhibitor. The inhibitor in boiled or unboiled preparations was non-dialysable. Hyaluronidase ('Hyalase', Benger Laboratories Ltd.) added in a concentration of 100 Benger units/ml. to boiled or unboiled suspensions of infant rat liver, buffered with acetate to pH 5·2, caused no fall in inhibitory power after 2 hr. incubation. Similar experiments with adult liver showed no acceleration of the destruction of inhibitor by hyaluronidase.

Glucuronidase in mouse liver (Walker & Levvy, 1951; Walker, 1952) could be brought completely into solution without enzyme inactivation by shaking suspensions with Ballotini, Grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10–30 min., or by treating the liver with water in the Waring Blendor for 3 min. Increasing the period of grinding the tissue with water in the glass homogenizer from 1 to 5 min. caused little change in the distribution and activity of the enzyme. Grinding the liver in acetone (glass homogenizer) followed by resuspension of the dry powder in water gave most of the enzyme in a soluble form, with only small losses in overall activity.

Table 4 shows the results obtained in similar experiments with rat liver. Here the possible effects of the treatment on the inhibitor had to be considered. With respect to the enzyme, the action of acetone in rat liver was the same as in mouse liver, but it had the additional effect of destroying the inhibitor in rat liver. The inhibitor was not found in the residue from the evaporated acetone extract. Unlike the enzyme in mouse liver, glucuronidase in rat liver showed marked inactivation after short periods of treatment in the Waring Blendor. A large part of the remaining enzyme was in a soluble state, and there appeared to have been some destruction of the inhibitor. Increasing the period of treatment with water in the glass homogenizer from 1 to 5 min. had little effect on either the enzyme or the inhibitor in rat liver.

In experiments with separate preparations of rat-liver enzyme and inhibitor (Table 5), the action of the Waring Blendor in destroying both was confirmed. The effects of shaking with Ballotini in the Mickle tissue disintegrator were even more striking.

Mode of action of the inhibitor. In the experiments described in this and the next section, preparations of enzyme-free inhibitor and inhibitor-free enzyme only were used, and assays were done in acetate buffer in absence of Triton X-100.

Table 4. *The effects of mechanical disintegration and acetone on the glucuronidase activity of adult rat liver (hooded Lister strain)*

(Samples of a single liver treated as shown. Activities measured in presence and absence of 0·075% Triton X-100 before and after fractionation with acetate buffer, pH 5·2.)

		G.U./mg. liver			
		Total activity		Soluble enzyme	
Treatment	Period (min.)	With Triton	Without Triton	With Triton	Without Triton
Glass homogenizer and water	1*	32·8	19·6	21·3	21·6
	5	32·4	20·5	22·0	22·0
Glass homogenizer and acetone†	1	30·2	28·8	26·7	27·0
Waring Blendor and water	2	27·0	23·4	22·5	22·6
	5	22·2	20·0	19·1	19·0

* Provides control for other experiments. † Dry powder resuspended in water.

Fourfold variation in the concentration of the rat-liver enzyme caused no significant change in the percentage inhibition observed on adding rat-liver inhibitor in a concentration of either 0·0125 or 0·0625% (w/v) in the assay tube. Fig. 2 shows the effect of increasing inhibitor concentration at fixed

Table 5. *Inactivation of rat-liver enzyme and destruction of inhibitor by mechanical treatment*

(Hooded Lister strain rats were used. Preparations of boiled, enzyme-free inhibitor and acetate-soluble, inhibitor-free enzyme from adult liver were treated and tested separately. Inhibitor tested with untreated enzyme. Assay in acetate buffer, pH 5·2, in absence of Triton X-100.)

Treatment	Period (min.)	Residual enzyme activity (G.U./mg. liver)	Action of treated inhibitor (% inhibition)
None	—	19·2	37
Waring Blendor	2	16·8	24
	5	13·0	13
	10	10·4	7
Tissue disintegrator	2	11·6	17
	5	2·9	6
	10	1·2	3

enzyme and substrate concentration. Inhibition increased to a maximum, always well below 100%, which was characteristic of the tissue of origin of both inhibitor (Fig. 2*a*) and enzyme (Fig. 2*b*). Two points are particularly noteworthy. A given inhibitor preparation had a greater effect on infant- than on adult-rat-liver enzyme. Adult-rat-liver enzyme was powerfully inhibited by boiled preparations of mouse liver and kidney. We have already seen (Table 3) that the enzyme in suspensions of these two mouse tissues was little affected by endogenous inhibitor. Mouse-liver enzyme was not appreciably affected by inhibitor preparations from the same tissue in concentrations in the assay as high as 5% (w/v), nor by inhibitor prepared from

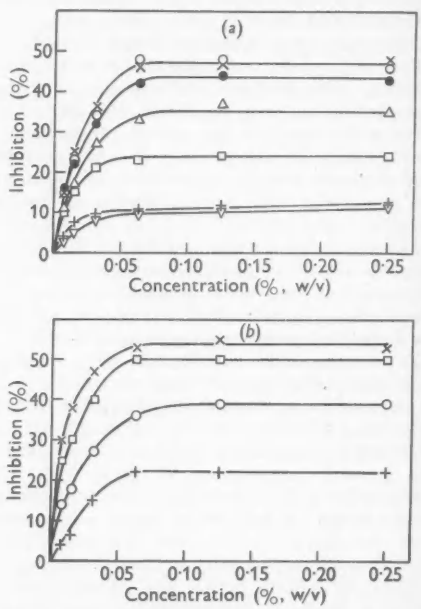

Fig. 2. Effect of varying inhibitor concentration with (*a*) a single preparation of adult-rat-liver enzyme and inhibitor of varied origin and (*b*) a single preparation of adult-rat-liver inhibitor and enzyme of varied origin. Inhibitor concentrations shown in terms of boiled tissue in the assay tube. Hooded Lister strain rats were used. Assay in acetate buffer, pH 5·2. Substrate concentration 0·00125M. ×, infant rat liver; ○, adult rat liver; □, adult rat kidney; +, adult rat spleen; ●, adult mouse kidney; △, adult mouse liver; ▽, adult mouse spleen.

any of the other tissues studied, at a concentration of 0·25%.

That pH was not a factor in the action of the tissue inhibitor was shown by experiments with

enzyme and inhibitor prepared from adult and infant rat liver. Identical figures for percentage inhibition were obtained in assays done in acetate buffer at pH 4·5 and 5·2. Inhibitor sedimented in acetate buffer, as already noted, adsorbed small amounts of soluble enzyme (never more than 10% of the total activity). In presence of substrate, adsorption increased to as much as 40% of the total soluble enzyme activity. However, adsorption by rat-liver inhibitor increased just as much with mouse-liver as with rat-liver enzyme, although in the former case there was no inhibition of adsorbed enzyme. While adsorption may well have been important in the mechanism of inhibition, the final effect was thus determined by other factors.

The possibility was considered that in those tissues in which it is affected by endogenous inhibitor, glucuronidase might exist in two forms, only one being open to inhibition. A variety of experiments was done with rat liver to test this possibility. Preparations of soluble enzyme were divided into fractions of approximately equal activity by making them 38% saturated with ammonium sulphate (cf. Mills, 1948; Kerr, Campbell & Levvy, 1949). Suspensions were divided into soluble and insoluble enzyme fractions and the latter brought into solution by incubation in acetate buffer. In neither case was any evidence obtained for differences in the response of the various fractions to a given inhibitor preparation.

Becker & Friedenwald (1949) found that rat-liver glucuronidase was inhibited up to a limit well below 100% by heparin and by impure hyaluronic acid. The effects of the two inhibitors were not additive. A study was made of the inhibition of acetate-soluble adult-rat-liver enzyme by powdered heparin (B.P., British Drug Houses Ltd.) and by a highly purified preparation of chondroitin sulphate (presented by Dr R. B. Duff). Fig. 3 shows that inhibition in both cases tended to a maximum which was well below 100% and was greatly increased by decreasing the pH of assay from 5·2 to 4·5. With neither compound was inhibition reversed by Triton X-100. Further experiments with heparin showed that it inhibited mouse-liver as strongly as rat-liver glucuronidase, and that it was as effective in citrate as in acetate buffer (cf. Fig. 1). Table 6 shows that the actions of heparin and chondroitin sulphate on rat-liver enzyme were additive to that of the endogenous inhibitor, but not to each other. Heparin, the more powerful inhibitor, appeared to displace chondroitin sulphate completely from combination with the enzyme.

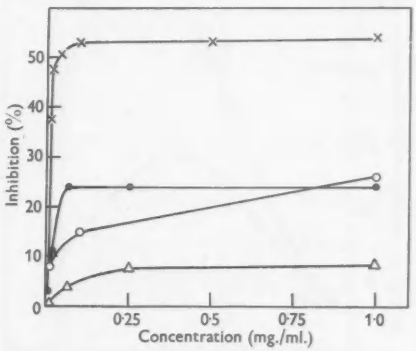

Fig. 3. Inhibition of adult rat-liver enzyme (hooded Lister strain) by varying concentrations of heparin and chondroitin sulphate at pH 4·5 and 5·2 (acetate buffer). Inhibitor concentrations expressed as mg./ml. in assay tubes (1 mg. heparin = 94·95 i.u.). ×, heparin at pH 4·5; ○, heparin at pH 5·2; ●, chondroitin at pH 4·5; △, chondroitin at pH 5·2.

The effect of varying substrate concentration. Fig. 4 shows the effect of varying the substrate concentration on the rate of hydrolysis of phenolphthalein glucuronide by acetate-soluble, adult-rat-liver glucuronidase in presence and absence of a fixed concentration of boiled inhibitor from the same liver. Inhibition increased with increasing substrate concentration, suggesting that the inhibitor combined with the enzyme-substrate complex, rather than

Table 6. *Inhibition of adult-rat-liver glucuronidase (hooded Lister strain) by boiled endogenous inhibitor from the same tissue (0·125%, w/v), heparin (0·5 mg./ml.) and chondroitin sulphate (0·5 mg./ml.), singly and in combination*

(Assay in acetate buffer, pH 4·5. Residual enzyme activity expressed as G.U./ml. acetate-soluble enzyme preparation. In parenthesis opposite each addition, the residual activity is shown in each column as a percentage of that for the control system.)

	Residual activity (G.U./ml. enzyme)			
Addition	Enzyme	Enzyme + endogenous inhibitor	Enzyme + heparin	Enzyme + chondroitin sulphate
None (control)	132	65	60	96
Chondroitin sulphate	96 (73%)	49 (75%)	60 (100%)	—
Heparin	60 (45%)	29 (45%)	—	60 (63%)
Endogenous inhibitor	65 (49%)	—	29 (48%)	49 (51%)

with the enzyme alone, a conclusion supported by the results of the experiments mentioned above on the adsorption of enzyme by inhibitor. This uncommon type of inhibition has been described by Burk (quoted by Ebersole, Guttentag & Wilson, 1944), who called it 'uncompetitive'. The term 'combined inhibition' seems more apt.

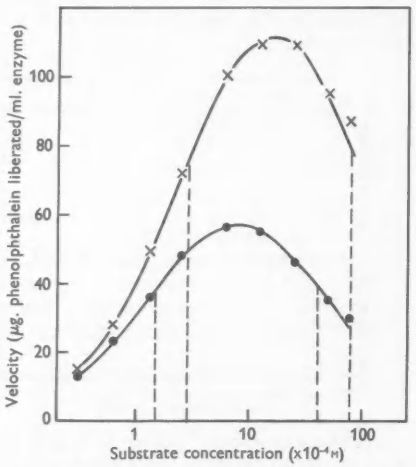

Fig. 4. The effect of varying the substrate concentration (S) on the rate of hydrolysis (v) of phenolphthalein glucuronide in presence (●) and absence (×) of 0·125% boiled tissue. Acetate-soluble enzyme from adult hooded Lister strain rat liver assayed in acetate buffer, pH 5·2. The points are experimental whilst the lines were calculated by substituting for K_s, K_2, n and V_m (Table 7) in the equation

$$v = \frac{V_m S}{S + K_s + S^n/K_2}.$$

The broken vertical lines show the values of K_s and K_2.

Inhibition by excess substrate, always pronounced with glucuronidase, became apparent at a lower concentration when the inhibitor was present. Each series of points in Fig. 4 was analysed by the method of Lineweaver & Burk (1934, case 3) for K_s, the dissociation constant of the active enzyme-substrate complex, K_2, the dissociation constant of the inactive complex, n, the number of substrate molecules per active enzyme centre in the inactive complex, and V_m, the theoretical maximum rate of hydrolysis for the particular preparation. The figures obtained for these constants are shown in Table 7, and the lines in Fig. 4 were drawn (taking $n = 2$) to fit the equation

$$v = \frac{V_m S}{S + K_s + S^n/K_2},$$

where v is the reaction velocity at different values of S, the substrate concentration. Table 7 also gives values for $\sqrt{(K_s K_2)}$, the substrate concentration for optimum enzyme activity. As well as a fall in V_m, the inhibitor caused an apparent increase in the affinity ($= 1/K_s$) of the enzyme for the substrate, but left n unchanged.

Table 7. *Kinetic constants for the hydrolysis of phenolphthalein glucuronide by rat-liver glucuronidase in presence and absence of endogenous inhibitor*

(Calculated by the method of Lineweaver & Burk (1934) from the results shown in Fig. 4.)

	Without inhibitor	With inhibitor
V_m (μg. phenolphthalein/ml. enzyme)	157	78
K_s ($\times 10^{-4}$ M)	2·9	1·5
n (±S.E.)	1·91 ± 0·08	1·94 ± 0·07
K_2* ($\times 10^{-4}$ M)	78	41
$\sqrt{(K_s K_2)}$† ($\times 10^{-4}$ M)	15	8

* Calculated by making $n = 2$ exactly.
† Substrate concentration for optimal velocity.

These results afford a striking demonstration of the way in which a tissue impurity can alter the kinetics of an enzyme reaction. The findings were confirmed in several other experiments with both adult- and infant-liver preparations. There was no significant difference between enzyme from adult and infant liver in values for K_s in absence of inhibitor. With a given enzyme preparation, the values of K_s and other constants in presence of inhibitor could be altered by altering the concentration of inhibitor added.

Inhibition by heparin of both rat- and mouse-liver glucuronidase was essentially non-competitive at pH 4·5 (acetate buffer). In non-competitive inhibition, V_m is the only constant to alter. The nature of the inhibition by chondroitin sulphate was not studied.

Fractionation of rat-liver glucuronidase. In all subsequent experiments, Triton X-100 was added during the enzyme assay, so that the effects of endogenous inhibitor were abolished. Table 8 shows the partition of glucuronidase activity observed after homogenizing samples of a single adult or infant rat liver in water and in 0·25 M-sucrose solution. In general, the results were similar to those previously obtained for the fractionation of mouse-liver glucuronidase (Kerr & Levvy, 1951; Walker & Levvy, 1951; Walker, 1952). The distribution of enzyme activity between the soluble and insoluble fractions was the same after both methods of fractionation employed. In the isotonic medium, practically all the enzyme activity was associated with insoluble material. After homogenizing in water, about 60% of the activity in adult liver and about 80% of the activity in infant liver was soluble.

Table 8. *Fractionation of rat-liver glucuronidase on the high-speed centrifuge, and on the low-speed centrifuge after buffering with acetate to* pH 5·2

(Hooded Lister strain rats were used, and the assay was done in acetate buffer, pH 5·2, in presence of Triton X-100.)

	G.U./mg. liver				
		High-speed centrifuge		Acetate treatment	
Homogenizing fluid	Total	Soluble	Insoluble*	Soluble	Insoluble
Adult female					
Water	36·8	22·7	14·1	21·2	14·8
0·25 M-Sucrose	35·6	1·9	33·7	1·4	34·3
Infant (4 days old)					
Water	17·2	13·9	3·3	13·0	4·3
0·25 M-Sucrose	17·0	1·4	15·6	1·0	16·4

* Calculated—see Experimental section.

It would appear that in rat liver, as in mouse liver, practically all the glucuronidase activity is initially associated with subcellular particles, from which it is released in hypotonic media. Suspensions of rat liver in water and in isotonic sucrose solution were examined by phase-contrast microscopy. The preparations were very similar to those of mouse liver (Walker & Levvy, 1951; Walker, 1952). Complete sedimentation of the insoluble enzyme was associated with the removal from the suspension of all but a very few of the smallest visible particles: buffering with acetate led to agglutination of all the visible components. All components of water suspensions, except the fat globules, displayed severe osmotic swelling.

The variation in rat-liver glucuronidase activity with the state of proliferation of the tissue. It would appear that the best measure of rat-liver glucuronidase activity is that obtained by the use of the whole suspension in conjunction with Triton X-100. It will have been noted that in previous experiments the activity of hooded Lister strain infant (4-day-old) liver was less than that of adult under these conditions of assay. Fig. 5 shows the change in rat-liver glucuronidase activity throughout the first months after birth for both hooded Lister and albino Wistar animals. With the hooded Lister strain, there was a sixfold rise in liver enzyme activity with age in males, and rather more in females: in females the activity remained constant, but in males it fell after 2 months to rather less than 75% of the female level. Albino Wistar rats differed from the hooded Lister strain in that the liver enzyme activity was much higher immediately after birth and showed a fall in females as well as males after 1 month. Apart from the higher glucuronidase activity in infants, water suspensions of Wistar rat liver behaved identically to those of hooded Lister rat liver of the same age as regards the fractionation of the enzyme and its activation by Triton X-100, and the stability of the inhibitor to incubation in acetate buffer in infants as compared with adults (cf. Fig. 1).

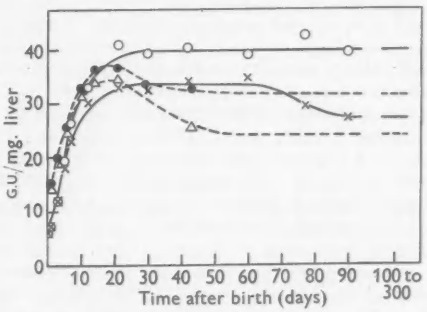

Fig. 5. Variation in hooded Lister and albino Wistar rat-liver glucuronidase activity with age. Assay in acetate buffer, pH 5·2, in presence of Triton X-100. Five Lister or three Wistar rats were used at each point. For definition of G.U., see text. ○—○, hooded Lister females; ×—×, hooded Lister males; ●---●, Wistar females; △---△, Wistar males.

Table 9. *Effect of partial hepatectomy on rat-liver glucuronidase activity (hooded Lister strain)*

(Assay in acetate buffer, pH 5·2, in presence of Triton X-100. Values shown as mean ± S.E. followed in parenthesis by the number of experiments.)

	G.U./mg. liver	
Days after operation	Males	Females
Control	27·3 ± 1·14 (15)	39·9 ± 1·45 (11)
1 to 4	23·3 ± 0·95 (12)	33·3 ± 1·51 (9)
6 to 10	28·7 ± 0·97 (6)	40·0 ± 1·63 (6)

Table 9 shows the effect of partial hepatectomy on liver glucuronidase activity. In both sexes there was a small, but real ($P < 0·05$), fall 1–4 days after operation, followed by a return to normal after 6–10 days.

DISCUSSION

Although there were minor points of difference, the factors which determined the distribution of glucuronidase in suspensions of rat liver were in general the same as those found for mouse liver (Kerr & Levvy, 1951; Walker & Levvy, 1951; Walker, 1952). After homogenizing the tissue in an isotonic medium, the enzyme was almost entirely associated with the subcellular particles. Homogenizing the tissue in water made a large part of the enzyme activity soluble, and this fraction could be further increased, notably by adding the surface-active agent, Triton X-100, or by incubation in acid buffers. The effects of these measures, and the contrast between the behaviour of β-glucuronidase and of more complex enzyme systems have been discussed in connexion with the enzyme in mouse liver (Walker & Levvy, 1951).

There was an important difference between the enzymes in rat and mouse liver in that the former were powerfully inhibited by an unidentified tissue constituent, apparently similar to the blood plasma anti-glucuronidase briefly described by Fishman, Altman & Springer (1948). The inhibitor in rat liver resembled heparin and hyaluronic acid, also inhibitors for the enzyme (Becker & Friedenwald, 1949), in that it was non-dialysable and did not cause complete inhibition. However, the action of the endogenous inhibitor on glucuronidase was very different from that of heparin. Chondroitin sulphate, which was also found to cause inhibition, behaved like heparin. Triton X-100 overcame the effect of the endogenous inhibitor, but not that of heparin or chondroitin sulphate. Hyaluronic acid was not studied, but the endogenous inhibitor appeared to be distinct from this compound since it was not destroyed by hyaluronidase, and since its action was additive to that of heparin, which is not true of hyaluronic acid (Becker & Friedenwald, 1949). Nevertheless, the properties of the endogenous inhibitor were not inconsistent with the possibility that it was mucopolysaccharide in character.

Two types of tissue preparation have been used for glucuronidase assay. Prior to the introduction of phenolphthalein glucuronide as substrate (Talalay *et al.* 1946), assay procedures made use of buffered extracts of soluble material, separated from cell debris after varying periods of incubation at slightly acid pH. With phenolphthalein glucuronide it became possible to use the crude water suspension, and this course has been widely followed. Thus, Fishman and his collaborators stopped using buffered tissue extracts in all work published subsequent to 1946 (private communication from Dr W. H. Fishman). Sources of error in the use of tissue extracts, arising from incomplete extraction of insoluble enzyme, have been discussed in connexion with mouse liver (Kerr & Levvy, 1951), and, as can be seen from Fig. 1, apply equally to the use of rat-liver extracts. The action of the endogenous inhibitor does not come into the question with buffered tissue extracts, since if not destroyed by incubation it will in any case have been precipitated and removed with cell debris. Crude water suspensions, if not too concentrated, were found satisfactory for the assay of mouse-liver glucuronidase activity (Kerr & Levvy, 1951). This is not true of water suspensions of rat liver due to the presence of the endogenous inhibitor which varies in its effects according to the age of the animal. Addition of Triton X-100 overcame the action of the endogenous inhibitor and led to apparently satisfactory results with water suspensions of rat liver. It should, however, be remembered that tissue preparations may contain other inhibitors, such as heparin and chondroitin sulphate, which are not overcome by Triton X-100.

It is clear that a single method of glucuronidase assay may not yield comparable results with different species and tissues, nor even with a single tissue in different physiological and pathological states. For purposes of comparative tissue assay, however, it may be argued that the final reading should include the effects of such factors as the inhibitor in rat liver. On the other hand, it is by no means certain that this inhibitor has any effect *in vivo*. Moreover, it was shown in the case of mouse liver (Walker, 1952) that the better the subcellular particles in the suspension were preserved, the smaller was their glucuronidase activity. This effect, unlike that of the endogenous inhibitor in rat liver, could be overcome by increasing the substrate concentration. Taking everything into consideration, it would appear that the best one can do is to try to measure the full activity of all the enzyme in the tissue. Whether the physiological function of β-glucuronidase can be deduced from figures thus obtained is another matter.

In rats, the glucuronidase activity of liver from newborn animals and of liver regenerating after partial hepatectomy was found to be less than that of normal adult liver. So far as comparison is possible, the general findings seem to be in agreement with those of Mills, Smith, Stary & Leslie (1950) and Mills (1951). The present results for rat liver are in contrast to those obtained for mouse liver (see for example Walker, 1952), where tissue growth or regeneration was found to be associated with increased glucuronidase activity. Kerr, Campbell & Levvy (1950) found that liver glucuronidase activity was higher in 4-day-old than in adult rats. It can now be seen that this result was obtained because, as shown by the original records, albino Wistar rats were used, and because only the

soluble enzyme fraction in the water suspension was taken for assay. In Wistar rats, the total glucuronidase activity in infants soon reaches and exceeds the ultimate adult value, and in general infant liver has a greater percentage of the enzyme in a soluble form than adult.

SUMMARY

1. After homogenizing rat liver in isotonic sucrose solution, nearly all the glucuronidase activity was found in the subcellular particles. Homogenizing in water made a large part of the enzyme soluble, and it was brought completely into solution by the surface-active agent, Triton X-100.

2. The activity of soluble and insoluble rat-liver glucuronidase in water suspensions was greatly reduced by a non-dialysable endogenous inhibitor. Inhibition, which was overcome by Triton X-100, was also seen in rat kidney, but was very slight in mouse liver and kidney. Rat-liver enzyme was powerfully inhibited by mouse-liver and kidney preparations.

3. The inhibitor in rat liver was thermostable at neutral pH. It was not completely sedimented on the high-speed centrifuge, but was completely precipitated by buffering to pH 5·2. Incubation in acetate buffer, pH 5·2, destroyed the inhibitor in adult-, but not infant-liver preparations.

4. Inhibition varied directly with substrate and inhibitor concentration, and the kinetics were analysed. The term 'combined inhibition' is suggested for this effect.

5. Inhibition by heparin and chondroitin sulphate was studied. The effects differed in important respects from those of the endogenous inhibitor.

6. The glucuronidase activity of newborn rat liver and of rat liver regenerating after partial hepatectomy was less than that of normal adult rat liver.

7. Sources of error in the assay of β-glucuronidase are discussed, with particular respect to the effects of the endogenous inhibitor in rat liver.

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The Antibacterial Effects of Substances Structurally Resembling Maleimide

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A series of structurally related compounds, including unsaturated cyclic imides, unsaturated dicarboxylic acids, and derivatives of 1:4-quinones has been investigated by Friedmann, Marrian & Simon-Reuss (1948*a*, *b*, 1949), and their action in inhibiting mitoses in cultures of chick fibroblasts was found to parallel their reactivity towards sulphydryl groups. Since quinones are well known to have some antibacterial effect (Cooper, 1913; Cooper & Nicholas, 1927; Oxford, 1942*a*, *b*; Oxford & Raistrick, 1942; Armstrong, Spinke & Kahnke, 1943; Barber, 1944; Atkins & Ward, 1945; see also Hoffman-Ostenhof, 1947); it seemed of interest to assay the whole group for antibacterial action.

EXPERIMENTAL AND RESULTS

The antibacterial effect was determined against a Gram-positive organism (*Staphylococcus aureus*, National Collection of Type Cultures (N.C.T.C.) 4163) and against a Gram-negative organism (*Escherichia coli*, N.C.T.C. 86) grown in a glucose broth medium at pH 6·8–7·0 containing the stated dilution of the compound under test. Tests were also carried out in the presence of 10% horse serum. The inoculum was one loopful of an 18–24 hr. subculture grown at 37°. The highest dilutions of the substances causing 50% inhibition of growth after 18 hr. at 37° are given in Table 1. For comparison the dilutions which cause a decrease of 50% in the mitoses of chick fibroblasts *in vitro* are also given (figures derived from Friedmann *et al.* 1948*a*, *b*, 1949 and unpublished). The figure in parenthesis after the name of a compound facilitates reference to Tables 1–3.

Another series of experiments was undertaken to decide whether the action of some of these compounds was bactericidal or bacteriostatic. Growth was observed over a period of 6 days and, after 3 and 6 days, portions from each dilution tube were removed and transferred to nutrient agar to see if any viable cells remained. The results are given in Table 2.

The effect of altered pH on the antibacterial activity of some of the quinones was also studied. The results are shown in Table 3.

Table 1. *Antibacterial effects of maleimide and related compounds*

(Dilutions of a 1/1000 soln. causing 50% inhibition of growth.)

		Staph. aureus		*Esch. coli*		
	Number and compound	Alone	+10% serum	Alone	+10% serum	Chick fibroblasts 50% inhibition of mitoses
1	Maleimide	1/720	1/50	1/950	1/240	(20% at 1/2000)
2	Citraconimide	1/15	(*a*)	1/30	(*a*)	1/5000
3	*N*-Ethylmaleimide	1/320	1/60	1/500	1/120	1/3700
4	Chloromaleimide	1/60	(*a*)	1/120	(*a*)	00 (*b*)
5	Succinimide	(*a*)	—	(*a*)	—	00
6	*N*-Ethyl-α-carboxymethylthiosuccinimide (*c*)	(*a*)	—	(*a*)	—	00
7	*N*-Ethylmaleimide + glutathione	(*a*)	—	(*a*)	—	Approx. 1/3700
8	Maleic acid	(*a*)	—	(*a*)	—	1/3000–1/2000
9	Citraconic acid	(*a*)	—	(*a*)	—	00
10	Fumaric acid	(*a*)	—	(*a*)	—	00
11	Mesaconic acid	(*a*)	(*a*)	(*a*)	—	00
12	Chloromaleic acid	(*a*)	—	(*a*)	—	00
13	Carboxymethylthiosuccinic acid (*d*)	(*a*)	—	(*a*)	—	1/700
14	*S*-1:2-Dicarboxyethylcysteine (*d*)	(*a*)	—	(*a*)	—	1/1200
15	*S*-1:2-Dicarboxyethylglutathione (*d*)	(*a*)	—	(*a*)	—	Approx. 1/200
16	1:4-Naphthaquinone	1/200	1/50	1/60	1/50	Approx. 1/63000
17	1:4-Dihydroxynaphthalene diphosphate	(*a*)	—	—	—	1/1000000
18	The same + phosphatase	1/60	1/20	—	—	—
19	1:4-Dihydroxynaphthalene mono(hydrogen succinate)	1/300	1/80	1/30	1/30	00
20	1:4-Dihydroxynaphthalene di(hydrogen succinate)	1/120	1/80	1/15	1/15	—
21	*S*-2(1:4-Naphthaquinonyl) thiolacetic acid	(*a*)	—	(*a*)	—	00
22	*S*-2(1:4-Naphthaquinonyl) glutathione	1/20	(*a*)	(*a*)	—	00
23	2-Methyl-1:4-naphthaquinone	1/300	—	1/30	—	00
24	2-Methyl-1:4-dihydroxynaphthalene diphosphate (*e*)	(*a*)	—	(*a*)	—	1/750
25	The same + phosphatase	1/50	1/8	—	—	—
26	2-Methyl-1:4-dihydroxynaphthalene di(hydrogen succinate)	1/90	(*a*)	1/15	(*a*)	1/430–1/850
27	The same + phosphatase	1/50	1/20	—	—	—
28	*S*-3(2-Methyl-1:4-naphthaquinonyl) thiolacetic acid	(*a*)	—	(*a*)	—	00
29	*S*-3(2-Methyl-1:4-naphthaquinonyl) glutathione	(*a*)	—	(*a*)	—	1/1000
30	2-Chloronaphthaquinone	1/200	(*f*)	(*f*)	(*f*)	—
31	*S*-3(2-Chloro-1:4-naphthaquinonyl) glutathione	(*a*)	—	(*a*)	—	00

(*a*) No inhibition at 1/10.
(*b*) 00, No mitotic inhibition at approx. 1/1700.
(*c*) The addition product of *N*-ethylmaleimide and thiolacetic acid (Marrian, 1949).
(*d*) Addition products of maleic acid with thiolacetic acid, cysteine and glutathione respectively (Morgan & Friedmann, 1938).
(*e*) Dicalcium salt.
(*f*) Irregular results obtained.

Table 2. *Bactericidal and bacteriostatic action of certain quinone derivatives, treated with alkaline phosphatase*

		Dilutions of a 1/1000 soln. causing inhibition of *Staph. aureus* after					
		18 hr.	40 hr.	3 days		5 days	
	Number and compound (phosphatase-treated)	50%	50%	50%	Complete destruction	50%	Complete destruction
26	2-Methyl-1:4-dihydroxynaphthalene di(hydrogen succinate)	1/150	1/100	1/90	1/64	1/90	1/64
	The same with 10% horse serum	1/25	1/25	1/25	—	1/25	—
24	2-Methyl-1:4-dihydroxynaphthalene diphosphate (dicalcium salt)	1/50	1/25	1/25	1/8	1/12	1/8
	The same with 10% horse serum and filtered	1/9	1/7	1/7	1/4	1/7	1/4
17	1:4-Dihydroxynaphthalene diphosphate	1/64	1/25	1/25	1/4	1/12	1/4?
	The same with 10% horse serum and filtered	1/12	1/6	1/6	1/2	1/6	1/2

Table 3. *Effect of* pH *variation on the antibacterial activity of some quinone derivatives*

			Dilutions of a 1/1000 soln. showing 50% inhibition of *Staph. aureus*	
	Number and compound	pH	18 hr.	4 days
27	2-Methyl-1:4-naphthaquinone di(hydrogen succinate) (phosphatase-treated)	6·0	1/150	1/50
		6·8	1/120	1/24
		7·3	1/120	1/50
25	2-Methyl-1:4-dihydroxynaphthalene diphosphate (dicalcium salt) (phosphatase-treated)	6·0	1/64	1/24
		6·8	1/28	1/12
		7·3	1/8	1/8
18	1:4-Dihydroxynaphthalene diphosphate (phosphatase-treated)	6·0	1/32	1/12
		6·8	1/50	1/24
		7·3	1/32	1/12

DISCUSSION

The compounds tested are best discussed in three groups: the imides, the unsaturated acids and the quinone derivatives.

Imides (1–6). These, the most interesting of the compounds tested, were notable for the comparatively high activity shown by the unsaturated members of the series against the Gram-negative *Esch. coli*, even when the medium contained 10% of horse serum, a potent source of sulphydryl material. In view of the known rapidity with which unsaturated cyclic imides add —SH groups (Friedmann *et al.* 1949; Marrian, 1949) across the unsaturated centre to form a saturated sulphur-substituted imide, it must be assumed that immediate fixation of the free imide is likely. In this connexion, the lack of activity shown by succinimide (5) and by the addition products (6, 7) of *N*-ethylmaleimide with thiolacetic acid and with glutathione is interesting. It should also be noted that addition of thiolacetic acid, but not of glutathione, destroys the antimitotic activity of *N*-ethylmaleimide and of maleimide (cf. 3 and 7) (Friedmann, Marrian & Simon-Reuss, 1952).

Compounds related to maleic acid (8–15). These show no antibacterial activity at the dilutions tested although a few of the series were mitotic inhibitors.

Compounds related to the quinones. In cases where antibiotic activity is shown the compounds are more active against Gram-positive organisms than against Gram-negative, showing marked dissimilarity to the imides in this respect, but in general any activity that is shown against the Gram-negative organism does not appear to be antagonized by horse serum. The pre-addition of —SH compounds to both 2-methyl-1:4-naphthaquinone (23) and to the unsubstituted naphthaquinone (16) give compounds (21, 22, 28, 29, 31) devoid of activity. The differences in activity shown by the water-soluble derivatives of the quinones, the diphosphates (17, 24) and the succinates (19, 20, 26) of the corresponding phenolic reduced substances are interesting. The more easily hydrolysed succinates showed approximately the same activity as the free quinone, but the diphosphates, being more stable,

were quite inactive until treated with alkaline phosphatase. Only partial activity is, however, restored. This confirms the earlier work of Atkins & Ward (1945).

Investigation (Table 2) as to whether the action of the quinone derivatives was bacteriostatic or bactericidal showed that, as expected, lower concentrations of the active compounds reduced growth but left bacteria viable, while higher concentrations caused complete destruction of the organism.

As the pH of the cultures varied from 6·0 to 7·3 the quinones tended to become somewhat less active.

There seems to be no obvious correlation in this series between antibiotic and antimitotic action.

SUMMARY

1. Certain unsaturated cyclic imides have been shown to have strong antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

2. The corresponding unsaturated dicarboxylic acids are inactive in this respect.

3. Earlier conclusions as to the antibiotic activity of quinones have been confirmed, and it has been shown that addition of sulphydryl compounds to the quinone destroys the activity. Increasing pH somewhat decreases the activity of the quinones.

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The Effect of Mastitis on the Carotenoids, Vitamin A and Phosphorus Compounds of Milk

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Of the various infections to which cattle are exposed, mastitis is one of the most prevalent and is the cause of great economic losses in all branches of milk production and manufacture (Davis & McClemont, 1939). Observations dating back many years have shown that mastitis is accompanied by definite changes in the chemical composition of milk. Amburger (1912) showed that protein, fat, lactose, ash, chloride and specific gravity were affected by mastitis, the chloride being affected in the opposite way to the lactose. About the same time other investigators showed similar changes in mastitic milk from sheep and goats (Grimmer, 1926). In the present paper studies of the effects of mastitis on phosphatase and on the partition of phosphorus, carotenoids and vitamin A are reported. At the same time results of analysis for other milk constituents are included, in order to show that the milk from the cows which were studied had its composition changed by the disease in the same way as had been found by earlier workers. Chanda & Owen (1949, 1951) found the changes in the partition of phosphorus produced by thyroxine and thiouracil treatment could be related to simultaneous changes of phosphatase. Accordingly, phosphatase was also studied in the present experiments.

Grimmer (1926) cites the observations of many earlier workers who studied the effects of mastitis caused by a variety of pathogens and of others who studied 'salty milk'. These early observers, among whom were Bögold & Stein (1890), noted that phosphate in the ash of milk became less as the proportion of sodium increased; and Grimmer remarks: 'Die Zusammensetzung der Asche nähert

sich somit derjenigen der Blutasche, die Milchdrüse hat scheinbar teilweise ihre Fähigkeit verloren, die ihr zugeführten Salze in passender Weise aufzunehmen, durch die erhöhten Chlornatriummengen wird der Salzgeschmack der Milch bedingt.' Accordingly, the blood of some normal and some mastitic cows was also studied in the present experiments.

EXPERIMENTAL

Eight Ayrshire cows were used, four controls (no. 1–4) and four suffering from mastitis (no. 5–8). In the controls there was no evidence of the presence of *Streptococcus agalactiae* or of micrococcal or staphylococcal mastitis, but in the four infected animals *Strep. agalactiae* was present in most of the quarters. The tests for the organisms were made by Mr A. L. Wilson, of the West of Scotland Agricultural College, Ayr. The degrees of infection recorded in the tables were assigned on the basis of chloride titres after it was known from bacteriological tests that *Strep. agalactiae* was present.

Methods of analysis. The total N of the milk was determined by the Kjeldahl method, and non-casein N by the procedure of Rowland (1938). The difference between them gave the casein N. Fat was estimated by the Gerber method (British Standards Institution, 1936), and total solids by drying a weighed sample of milk on a water bath followed by 3 hr. in an oven at 100°. The content of non-fatty solids was obtained by difference, but was calculated and recorded on the basis of fat-free milk. For estimating chloride the method of Davies (1938) and for phosphatase and the partition of phosphorus the procedures used by Chanda & Owen (1951) were adopted. Total thiamine and the partition of thiamine were determined by a slight modification (Chanda, McNaught & Owen, 1952) of the method described by Houston, Kon & Thompson (1940). The reading for each fraction was made in a Spekker fluorimeter in *iso*butanol as thiochrome (Jansen, 1936). For the determination of the partition of carotenoids and vitamin A in milk, the fat was extracted by the method of Olson, Hegsted & Peterson (1939). The chromatographic separation of β-carotene from other carotenoids and of vitamin A alcohol from vitamin A ester were made in the extracted fat by a modification (Chanda, Owen & Cramond, 1951; Chanda & Owen, 1952*a*) of Ganguly, Kon & Thompson's (1947) modification of Müller's (1944) method. Both the total carotenoids and the β-carotene were determined by measuring their extinctions at 451 mμ. in a Unicam spectrophotometer. In calculating the potency it was assumed that $E^{1\%}_{1\,cm.}$ in *n*-hexane for both the mixed carotenoids and β-carotene was 2500. All the E readings for the vitamin A fractions were corrected by the three-point method of Morton & Stubbs (1946), 315, 328 and 337 mμ. being used as fixation points. The corrected E value at 328 mμ. was multiplied by the conversion factor 1900 to calculate the potency in international units (World Health Organization: Expert Committee on Biological Standardization, 1949). When calculating the potency on a weight basis, 1 i.u. was taken as equivalent to 0·3 μg. vitamin A alcohol.

Blood. The partition of carotenoids and vitamin A in blood serum was made by the same chromatographic procedure as for milk fat (Chanda & Owen, 1952*a*). The extraction of blood serum lipids, together with carotenoids and vitamin A, was as described by Yudkin (1941) but *n*-hexane was used as the solvent. The correction procedure of Morton & Stubbs (1946) was applied for the final estimation of vitamin A ester or vitamin A alcohol, and here again the conversion factor 1900 was used to calculate the potency. Cama & Goodwin (1949) have shown that the estimation of serum vitamin A by the three-point correction of the ultraviolet extinctions agrees with that obtained by the $SbCl_3$ method.

RESULTS

The fat, non-fatty solids, chloride and phosphatase contents of the milk. Table 1 shows that, as found by most previous observers, the fat content of the milk was decreased by mastitis, while at the same time, and again in accord with previous observers, the non-fatty solids were decreased and chloride increased. As reported by previous investigators, mastitic infection was accompanied by an enhancement of the phosphatase activity of the milk (Table 2).

The ratio of nitrogen to phosphorus (N/P) *in the milk.* During the course of the experiment, cow 1 was in its 6th–10th week of lactation, whereas cow 2 was between its 16th and 20th weeks. The fact that the N/P ratio was higher for cow 2 (6·3 in Table 1) than for cow 1 (5·2) is consistent with the observation of Chanda & Owen (1952*b*) that the N/P ratio attains a minimum between the 2nd and 4th weeks post-partum and that thereafter it increases throughout lactation. For the milk from the individual quarters of the two control cows (Table 1) the N/P ratios were remarkably similar, but in the milk from the infected quarters of the other cows the ratios were much higher than in the uninfected quarters. Thus for the severely infected quarter of cow 6 the ratio was as high as 9·4 compared with 5·3 for the normal quarter. The maximum value previously obtained by Chanda & Owen (1952*b*) was 7·2 in the milk of cows in their 40th week of lactation. Somewhat higher values were obtained also for the milk of cows treated with thiouracil (Chanda & Owen, 1951) but no value as large as 9·4 was observed. It will be seen from Table 1 that the abnormal ratios were caused by both an increase in the nitrogen and a decrease in the P of the milk.

The partition of phosphorus. Table 1 shows that where the degree of infection was severe, as judged by the chloride values, the phosphatase content of the milk was high. Thus in cow 6, the phosphatase in the milk of the right hind quarter was about 4 times that of the right fore quarter, and the values for the milk of the left fore and left hind quarters were somewhat greater than for the milk of the healthy right fore quarter. The differences between the phosphatase titres of the milk of the four quarters of each of the two control cows were relatively small.

Chanda & Owen (1952*b*) observed that the changes in phosphatase as lactation progressed were inversely correlated with the ester P, lipid P and casein P. The partition of P recorded in Table 2

Table 1. *The effect of mastitis on the content of some of the principal constituents of the milk*

Cow		No. of samples	Quarter of udder*	Degree of infection	Fat (%)	Non-fatty solids in fat-free milk (%)	Chloride (mg./100 g.)	Nitrogen (mg./100 g.)	Phosphorus (mg./100 g.)	N/P
State	No.									
Normal	1	5	RF	–	3·6	8·76	78	592	115	5·1
		5	RH		4·2	8·81	85	578	111	5·2
		5	LF	–	3·5	8·79	81	595	114	5·2
		5	LH	–	4·0	8·72	75	575	112	5·1
	2	5	RF	–	3·6	8·45	117	581	94	6·2
		5	RH		3·5	8·39	115	596	95	6·3
		5	LF	–	3·9	8·54	124	567	90	6·3
		5	LH	–	3·2	8·48	113	591	92	6·4
Mastitis	5	1	RF	–	4·3	8·86	69	611	127	4·8
		1	RH	+	4·4	8·63	111	629	121	5·2
		1	LF	+ + +	2·9	7·94	180	716	102	7·0
		1	LH	+ + +	3·2	8·29	144	709	108	6·6
	6	4	RF	–	3·5	8·96	76	569	108	5·3
		4	RH	+ + +	1·7	7·58	185	694	74	9·4
		4	LF	+	2·8	8·54	112	611	98	6·2
		4	LH	+	2·9	8·65	117	625	95	6·6
	7	4	RF	–	3·3	8·81	85	591	109	5·4
		4	RH	+ +	2·6	8·32	122	654	93	7·0
		4	LF	+ + +	2·1	7·46	194	679	78	8·7
		4	LH	+ +	2·3	8·23	129	645	88	7·3
	8	4	RH	+	3·1	8·27	117	657	112	5·9
		4	LH	+ + +	1·8	7·54	198	725	77	9·4

* RF = right fore; RH = right hind. LF = left fore; LH = left hind.

Table 2. *The effect of mastitis on the partition of phosphorus in the milk*

Cow		No. of samples	Quarter of udder*	Degree of infection	Total P (mg./100 g.)	Total P as				Phosphatase (phenol units†)
						Inorganic	Ester	Lipid	Casein	
State	No.					(%)	(%)	(%)	(%)	
Normal	1	3	RF	–	112	58·4	11·7	11·2	18·7	64
		3	RH	–	107	59·8	11·2	10·9	18·1	75
		3	LF	–	110	59·2	11·6	11·4	17·8	69
		3	LH	–	108	60·3	11·3	10·7	17·7	78
	2	3	RF	–	96	66·7	7·2	10·2	15·9	129
		3	RH	–	98	65·5	7·6	10·5	16·4	121
		3	LF	–	93	66·9	7·0	10·0	16·1	134
		3	LH	–	95	66·2	7·2	10·3	16·3	127
Mastitis	5	1	RF	–	127	53·7	13·9	13·1	19·3	48
		1	RH	+	121	60·1	11·0	10·7	18·2	78
		1	LF	+ + +	102	74·5	4·6	7·6	13·2	189
		1	LH	+ + +	108	71·8	5·4	8·5	14·4	166
	6	3	RF	–	109	54·9	13·5	12·8	18·8	54
		3	RH	+ + +	72	75·3	4·2	7·7	12·8	202
		3	LF	+	99	61·5	10·8	10·5	17·2	80
		3	LH	+	95	62·5	10·5	10·7	16·3	92
	7	3	RF	–	107	61·7	10·7	10·9	16·7	87
		3	RH	+ +	91	65·9	7·5	10·4	16·2	115
		3	LF	+ + +	79	75·6	4·2	7·5	12·7	207
		3	LH	+ +	89	66·3	7·3	10·4	16·0	121
	8	2	RH	+	114	65·8	7·8	10·3	16·1	114
		2	LH	+ + +	76	75·7	4·3	7·1	12·9	229

* See Table 1 for explanation of symbols.

† See Chanda & Owen (1951).

shows that in mastitis there is a similar relationship; the changes in phosphatase were accompanied by changes in the partition of P. Thus in the milk of the severely infected right hind quarter of cow 6 the inorganic P formed 75% of the total P compared with 55% in the sound right fore quarter. The two left quarters which were only mildly infected also gave milk with more of the P in the inorganic form. It will be observed that the milk of control cow 2 was richer in phosphatase than that of cow 1, and that it had also a larger proportion of P in the inorganic form. In fact, as shown in Fig. 1, the inorganic P was positively correlated with phosphatase.

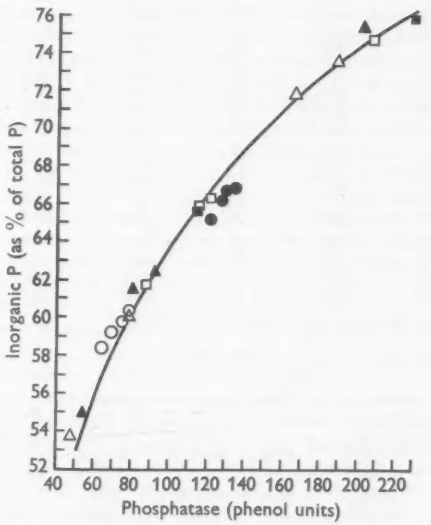

Fig. 1. The relationship between inorganic P, expressed as % of total P and phosphatase in normal and mastitic milk. Curve fitted by the method of least squares. ○, normal cow no. 1; ●, normal cow no. 2; △, mastitic cow no. 5; ▲, mastitic cow no. 6; □, mastitic cow no. 7; ■, mastitic cow no. 8.

Ester, casein and lipid P were greater in the normal quarters than in the infected quarters and all these fractions of organic P were negatively correlated with phosphatase. These correlations held whether the fractions were expressed as concentrations of P in the milk or as percentages of the total P. The negative correlation between phosphatase and ester P is recorded in Fig. 2, and that between lipid P and phosphatase in Fig. 3. Ester P, like phosphatase, and some other constituents, may be used to compare the severity of infection in different quarters of the same cow. Even the lowest ester P values corresponding to the highest

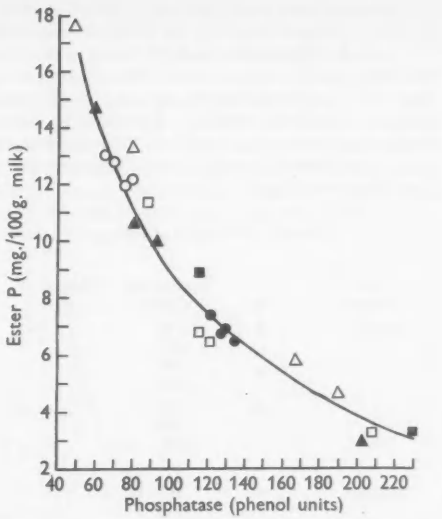

Fig. 2. The correlation between phosphatase and ester P in normal and mastitic milk. Symbols and cows are the same as those in Fig. 1.

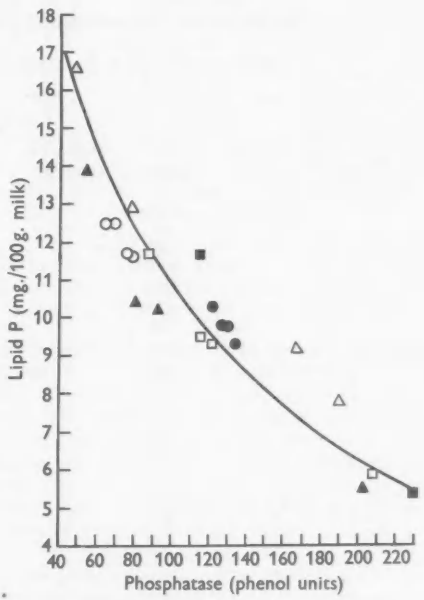

Fig. 3. The correlation between phosphatase and lipid P in normal and mastitic milk. Symbols and cows are the same as those in Fig. 1.

phosphatase titres observed in the most severely infected quarters are thus of limited diagnostic value, similar figures having been noted in the milk of cows treated with thiouracil (Chanda & Owen, 1951) and in cows towards the end of lactation (Chanda & Owen, 1952*b*). Nevertheless, large differences between the milks of different quarters of any one cow may well indicate the presence of some form of mastitis.

The ratio of N/P *in casein.* Both the casein N and the casein P of the milk were decreased by mastitis. The decreases in casein P were, however, much greater than those in casein N so that the ratio N/P for the casein was greatly increased in the milk from infected quarters (Table 3). For example, in cow 7 the ratio casein N/casein P was as large as 38 in the infected quarter (left fore) as compared with only 24 in the normal quarter (right fore). The ratio of

Table 3. *The ratio of nitrogen to phosphorus in the casein of milk affected by mastitis*

Cow		Quarter of udder*	Degree of infection	Total N (mg./100 g.)	Casein N (mg./100 g.)	Casein N as % of total N	Casein P (mg./100 g.)	N/P in casein
State	No.							
Normal	3	RF	–	581	412	71	20·4	20·2
		RH	–	577	414	72	20·1	20·6
		LF	–	584	418	72	19·9	21·0
		LH	–	572	423	74	20·5	20·6
	4	RF	–	567	407	72	16·4	24·8
		RH	–	586	434	74	17·6	24·7
		LF	–	574	427	74	17·9	23·9
		LH	–	579	422	73	17·5	24·1
Mastitis	5	RF	–	611	453	74	24·5	18·5
		RH	+	629	445	71	22·0	20·2
		LF	+ + +	716	422	59	13·5	31·3
		LH	+ + +	709	429	61	15·5	27·7
	7	RF	–	598	435	73	17·8	24·4
		RH	+ +	646	417	65	14·5	28·9
		LF	+ + +	694	388	56	10·2	38·0
		LH	+ +	656	421	64	14·2	29·6

* Only one sample from each quarter of each cow was analysed. Symbols as in Table 1.

Table 4. *The effect of mastitis on the partition of thiamine in the milk*

Cow		Quarter of udder*	Degree of infection	Thiamine (μg./100 ml. fat-free milk)		Total thiamine present as			Phosphatase (phenol units†)
						Free	Cocarboxylase	Protein-bound	
State	No.			Free	Total	(%)	(%)	(%)	
Normal	3	RF	–	23·2	39·7	58·5	28·7	10·8	58
		RH	–	23·9	40·4	59·2	29·5	9·5	56
		LF	–	23·3	38·6	60·4	27·4	10·2	61
		LH	–	22·6	39·2	57·7	29·9	10·6	53
	4	RF	–	23·6	32·5	72·6	16·9	8·7	134
		RH	–	24·5	34·6	70·7	18·8	8·9	131
		LF	–	24·8	33·9	73·1	17·1	8·3	136
		LH	–	22·7	32·1	70·8	18·5	9·2	129
Mastitis	5	RF	–	24·3	44·6	54·4	33·4	11·5	48
		RH	+	23·0	37·3	61·7	27·2	9·5	78
		LF	+ + +	23·9	28·6	83·6	8·6	5·6	189
		LH	+ + +	25·3	30·5	82·9	9·4	5·9	166
	6	RF	–	22·9	41·8	54·8	32·5	10·9	52
		RH	+ + +	22·9	27·2	84·2	8·8	5·5	198
		LF	+	21·9	35·5	61·8	26·6	9·4	83
		LH	+	21·4	34·4	62·2	26·1	9·7	89
	7	RF	–	23·7	38·4	61·6	26·7	9·3	82
		RH	+ +	22·6	32·9	68·7	21·9	7·5	118
		LF	+ + +	22·3	26·5	84·0	8·2	5·3	203
		LH	+ +	22·2	31·6	70·3	20·7	7·6	124
	8	RH	+	23·8	34·9	68·1	22·3	7·4	119
		LH	+ + +	22·0	25·7	85·4	7·9	5·1	221

* Two samples of milk were analysed from each quarter, except for the quarters of cow 5 for which only one sample was analysed. Symbols as in Table 1.

† See Chanda & Owen (1951).

N/P in the casein was remarkably constant in the different quarters of each of the two control cows, but cow 4, which was at a later stage of lactation than cow 3, showed a bigger ratio.

Phosphatase and the partition of thiamine. The results for the partition of thiamine are shown in Table 4 where the phosphatase titres for the same samples of milk are also recorded. The values for free thiamine were very similar for the milks from all the quarters of all six cows, in spite of considerable differences in the total thiamine. For example, in the milk of the two control cows there was a large difference in phosphatase titre, the larger titre in cow 4 corresponding to a smaller figure for total thiamine, but the absolute amount of free thiamine was the same for the milk of both cows, the decrease in the total thiamine occurring in the cocarboxylase and protein-bound forms. These relationships were even more apparent in the milks from individual quarters of the infected cows. Where the phosphatase was high in the milk of a particular infected quarter, the amount of free thiamine was the same as in the milk of the non-infected quarter of the same cow, but the total thiamine and the cocarboxylase and protein-bound forms were all markedly reduced. In the milks from the individual quarters of each of the two control cows there were no significant differences.

The fact that the percentage of the total thiamine which is present in the free form increases in the milk of infected quarters was noted earlier by Thompson (1945), but the present work goes further in showing the constancy of the values for free thiamine and the large decreases which can occur in the cocarboxylase and protein-bound forms. In the severely infected quarters of cows 5–7, the cocarboxylase was only 8 or 9% of the total, whereas for the normal quarters the corresponding figures were around 30%. There was thus an inverse relationship between cocarboxylase and phosphatase. The close but curvilinear inverse correlation between these two substances is shown in Fig. 4. The values for protein-bound thiamine in the milk of normal and infected quarters varied in much the same way as the values for cocarboxylase.

The positive correlation between the phosphatase titre and the percentage of the total thiamine present in the free form (Table 4) appears to be general. It was observed by Houston *et al.* (1940) for the milk of cows and goats, and by Braude *et al.* (1947) for sow's milk. Chanda *et al.* (1952) found it to exist in the milk of cows treated with thyroxine and thiouracil. The inverse relationship between phosphatase and phosphorylated forms of thiamine were also shown to hold for cow's milk throughout lactation (Chanda & Owen, 1952*b*), and also in the milk of cows treated with thyroxine and thiouracil (Chanda, McNaught & Owen, 1952). It appears therefore that there is a persistent relationship between phosphorylated compounds and phosphomonoesterase whether the changes are produced by mastitis (Table 4 and Fig. 4) or are induced artificially by hormones (Chanda *et al.* 1952) or are produced naturally during declining lactation (Chanda & Owen, 1952*b*). The inverse relationship between phosphatase and phosphorylated thiamine has also been demonstrated in human milk (Chanda *et al.* 1951) and in goat milk (Chanda, 1951).

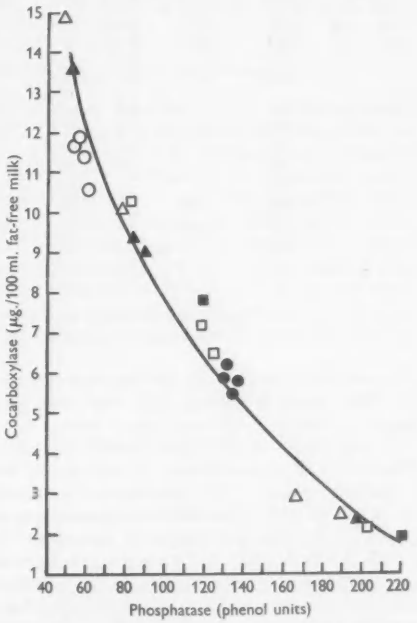

Fig. 4. The relation between phosphatase and cocarboxylase in normal and mastitic milk. ○, normal cow no. 3; ●, normal cow no. 4; △, mastitic cow no. 5; ▲, mastitic cow no. 6; □, mastitic cow no. 7; ■, mastitic cow no. 8.

The partition of carotenoids and vitamin A. Large increases in the total-carotenoid content of milk during clinical mastitis were reported by Thompson (1945). The results recorded in Table 5 show that, though the fat content of the milk was decreased by mastitis, the carotenoids were considerably increased in the milk from infected quarters. The percentage of the total carotenoids which was present as β-carotene was surprisingly constant and was not influenced by mastitis, but the content of β-carotene/g. fat was very markedly increased for the milk of infected quarters. Where the infection was greatest the amount of β-carotene/g. fat was 6 or 10 times as large as the corresponding figure for the milk of a normal quarter (Table 5), and even for

Table 5. *The effect of mastitis on the partition of the carotenoids and vitamin A in cows' milk*

Cow		Quarter of udder*	Degree of infection	Fat (%)	Total carotenoids (μg./100 ml.)	β-Carotene (μg./g. fat)	β-Carotene (As % of the total carotenoids)	Partition of vitamin A: Total (i.u./100 ml. milk)	Partition of vitamin A: Total (i.u./g. fat)	Partition of vitamin A: Alcohol (i.u./g. fat)	Partition of vitamin A: Alcohol (% of total)	Partition of vitamin A: Ester (% of total)	A/C†
State	No.												
Normal	3	RF	–	3·4	28·9	7·5	88	115	33·8	1·5	4·3	93·9	1·51
		RH	–	3·9	30·2	6·8	88	120	30·8	1·8	5·8	92·2	1·51
		LF	–	3·7	29·7	7·4	92	117	31·6	1·6	5·1	93·0	1·42
		LH	–	4·1	28·4	6·3	91	124	30·2	1·7	5·6	92·6	1·60
	4	RF	–	3·5	27·8	7·3	91	97	27·7	1·5	5·4	92·5	1·26
		RH	–	3·7	29·4	7·2	90	108	29·2	1·3	4·5	93·3	1·35
		LF	–	3·8	29·7	7·0	90	109	28·7	1·4	4·9	93·0	1·37
		LH	–	3·3	27·2	7·3	89	92	27·9	1·4	5·1	92·9	1·27
Mastitis	6	RF	–	3·6	29·5	7·4	90	118	32·8	1·9	5·7	92·3	1·47
		RH	+ + +	1·6	130·6	74·4	91	49	30·6	6·0	19·6	78·6	0·14
		LF	+	2·7	52·7	17·2	91	86	31·9	3·0	9·5	88·3	0·60
		LH	+	2·9	54·8	17·2	91	91	31·4	2·8	8·9	88·9	0·61
	7	RF	–	3·2	28·4	8·0	90	95	29·7	1·7	5·7	92·3	1·24
		RH	+ +	2·5	58·7	20·9	89	76	30·4	3·5	11·6	86·5	0·48
		LF	+ + +	2·1	125·2	53·5	90	66	31·4	6·7	21·2	77·2	0·20
		LH	+ +	2·4	63·6	24·1	91	74	30·8	3·9	12·5	85·6	0·43
	8	RH	+	3·0	55·9	16·6	89	92	30·7	3·3	10·8	87·3	0·61
		LH	+ + +	1·9	123·4	58·7	90	59	31·1	5·4	17·6	80·6	0·18

* Three samples of milk were analysed for each quarter. Symbols as in Table 1.
† A/C is the ratio of vitamin A to β-carotene, both expressed as μg./g. fat.

mildly infected quarters the increase was considerable. There was, however, little variation from quarter to quarter in the two control cows.

Table 5 shows that the total vitamin A content, unlike the carotene content of the milk, was decreased by mastitis. The decreases in vitamin A were, however, proportional to the decreases in the fat content so that the vitamin A content of the milk fat remained practically unchanged. In cow 6 the vitamin A content was only 49 i.u./100 ml. milk in the most infected (right hind) quarter as compared with 118 i.u./100 ml. in the sound (right fore) quarter, but the corresponding values expressed as i.u./g. fat were 31 and 33 respectively. This small difference in the vitamin A content of the fat cannot be considered of any significance since, in cow 7, one of the infected quarters had 1·7 i.u. more vitamin A/g. fat than its own control quarter, and there were differences of the same order in the values for the different quarters of the control cows. The decreases in the total vitamin A content of the milk from infected quarters which accompany the simultaneous decreases in the fat content are consistent with the observation of Thompson (1945), but are in contrast to that of Miller, Lease & Anderson (1940) who reported an increase in the vitamin A content in the milk from the infected quarter of a cow in which mastitis was artificially produced.

The vitamin A content of the milk fat of control cow 3 was higher than for that of control cow 4. This agrees with the earlier finding of the present author (Chanda, 1953) that the vitamin A content of cow and goat milk fat, in contrast to that of the carotenoids, continues to change throughout lactation. In these earlier experiments Chanda (1953) showed that variations in the ratio of vitamin A/g. fat to β-carotene/g. fat (A/C) for the milk fat of different cows was determined by the vitamin A content, since the carotene content of the fat was constant as long as the diet remained unchanged. In the present experiments the A/C ratios recorded in Table 5 show that control cow 4 which was at about the 25th week of lactation gave a slightly smaller A/C ratio than control cow 3 which was at an earlier stage of lactation, and that the difference was caused by differences in the vitamin A contents of the fat. When, however, as in mastitis, the A/C ratio in any one cow varied widely from quarter to quarter it was the carotene and not the vitamin A which determined the variation in the A/C ratio. The smallest ratio of 0·14 was recorded in the very severely infected quarter of cow 6 corresponding to the largest β-carotene figure. As in normal milk there was no correlation between the vitamin A and the β-carotene contents of the fat of mastitic milk, but a close negative correlation was found between β-carotene and the A/C ratio.

In normal milk only a small percentage of vitamin A is present as alcohol (Parrish, Wise & Hughes, 1947; Ganguly *et al.* 1947; Chanda & Owen, 1952*a*). This was confirmed in the present experiments for

Table 6. *The partition of carotenoids and vitamin A in the blood serum of normal and mastitic cows*

Cow		Total lipid (mg./ 100 ml.)	Total carotenoids (μg./ 100 ml.)	β-Carotene (% of total caro-tenoids)	β-Carotene (μg./g. lipid)	Vitamin A Alcohol (i.u./ 100 ml.)	Vitamin A Ester (i.u./ 100 ml.)	Vitamin A Total (μg./g. lipid)	A/C* × 10³
State	No.								
Normal	1	367	1329	91	3621	105	39	131	36·1
	2	349	1213	88	3476	98	32	123	25·4
	3	356	1465	91	4115	96	43	129	31·4
	4	334	1396	90	4180	102	30	132	31·5
Mastitis	6	388	1152	89	2969	97	37	116	39·1
	7	375	1097	88	2925	95	30	112	38·3
	8	392	1194	88	3046	103	36	117	38·5

* A/C is the ratio of vitamin A to β-carotene, both expressed as μg./g. lipid.

individual quarter samples (Table 5). For the two control cows the vitamin A alcohol ranged between 4 and 6 % of the total vitamin A, whereas in mastitis, when the total vitamin A content of the milk was considerably decreased, both the percentage present as alcohol and the actual amount of the alcohol form were increased (see also Ganguly *et al.* 1947). For example, in the normal (right fore) quarter of cow 7 the amount of vitamin A alcohol was found to be 5·4 i.u./100 ml. milk, while the value for the severely infected quarter of the same cow was 14 i.u. The alcohol form of vitamin A in the milk increased therefore by 159 % while the total vitamin A (Table 5) was decreased by 30 %. Similar results were obtained for cow 6. It will be noted also from Table 5 that the content of vitamin A ester in the milk of mastitic quarters was considerably less than in the milk of normal quarters.

The partition of carotenoids and vitamin A in the blood serum. The results for the partition of carotenoids and vitamin A determined in four normal cows and three infected cows are recorded in Table 6. They suggest that the lipid content of the serum of mastitic cows was slightly greater than that of the normal cows. This is in contrast to the corresponding results observed for the fat content of the milk. Total carotenoids were consistently less in the serum of the mastitic cows than in that of the normal cows, but there were no significant differences in the percentages of total carotenoids present as β-carotene. The contents of β-carotene in the blood-serum lipids of mastitic cows were much smaller than those for the normal cows. The partition of vitamin A showed that the alcohol form of vitamin A in the serum varied within the range of 96 and 105 i.u./ 100 ml. (Table 6), and that there was no difference in this respect between the normal and the infected cows. In confirmation of earlier findings (Chanda, 1953) it was observed that the blood serum of grazing cattle contained vitamin A ester, but again there was no difference between the vitamin A ester values of the normal and of the infected cows. Since the β-carotene content of the serum of mastitic cows was smaller than for the normal animals, the ratio of vitamin A to β-carotene (A/C) tended to be greater in the mastitic cows than in the normal cows. The A/C ratio for the blood serum, was, however, much smaller than in the milk fat, and the A/C ratio in the milk fat of mastitic quarters lay between the ratio for the serum lipid and for the normal milk fat from the same cow. Thus a typical value for normal milk fat was about 1·4 (Table 5); for milk from a mastitic quarter it was on one occasion as low as 0·14 (Table 5), and for blood serum it was about 0·036 (Table 6).

DISCUSSION

Variations in the composition of milk

The composition of the blood and milk taken at the same time from the four normal cows used in the present work is recorded in Table 7, from which it can be seen that those constituents like nitrogen and chloride, which are present in the blood serum in greater amounts than in the milk, were increased by mastitis. Fat, phosphorus and thiamine, which are present in smaller amounts in the blood than in the milk, showed large decreases in mastitis. This generalization does not, however, apply to phosphatase which was increased by mastitis in spite of the fact that it was present in lesser amount in the blood serum of the cow. It is known that the mammary gland itself is strikingly rich in phosphatase (Folley & Kay, 1935) and it is probable that the milk phosphatase originates in the mammary gland itself.

Chanda & Owen (1951) showed that the variation in the ratio of N/P is an index of the activity of the mammary gland, for when the activity of the gland was artificially enhanced by thyroxine treatment, the ratio was increased. By contrast, thiouracil had the opposite effect. The decreased activity of the mammary gland in mastitis is also reflected here by the larger N/P ratio in the milk of infected quarters (Table 1). Chanda & Owen (1951) showed that the phosphorus content of casein can vary considerably

Table 7. *The average concentration of some constituents in the milk and blood serum of four normal cows*

Constituent (per 100 ml.)	Blood serum	Milk	Increased (+) or decreased (−) in the milk from quarters infected with mastitis
Fat (mg.)	350	3685	−
Nitrogen (mg.)	1227	587	+
Chloride (mg.)	333	98	+
Phosphorus (mg.)	17	102	−
Ester P (mg.)	2	10	−
Phosphatase (phenol units)*	21	98	+
Thiamine (μg.)	14	36	−
β-Carotene (μg.)	1206	27	+
Vitamin A (i.u.)	134	108	−

* See Chanda & Owen (1951).

and that it tends to increase during thyroxine treatment and decrease with thiouracil. In the present experiments this variation in the phosphorus content of casein was very evident in mastitic milk (Table 3). The casein fraction of the milk from infected quarters was poorer in phosphorus than that from normal quarters. This difference in composition is in accord with the well-known fact that it is difficult to prepare casein of constant phosphorus content (Rogers, Associates of, 1935). Even in the two normal cows which were at different stages of lactation, the ratio of N/P in the casein was significantly different (Table 3). The theory of Synge (1950) on the existence of families of nearly identical proteins is compatible with the present observations on the differences in the composition of casein. The present results are also consistent with the recent observations of Warner (1944), of Hipp, Groves, Custer & McMeekin (1952) and of Dovey & Campbell (1952) who confirmed the earlier observation of Mellander (1939) and found three distinct components in casein. Hipp *et al.* (1952) showed further that the phosphorus content of the three components differed, but that their nitrogen contents were remarkably similar. The larger differences shown in Table 3 for the ratios of N/P in casein may therefore reasonably be attributed to changes in the proportion of the three components of casein when a quarter is infected.

Carotenoids and vitamin A in the blood serum and the milk

Normally the passage of carotenoids into the milk, unlike that of vitamin A, is very limited (Deuel, Hallman, Johnston & Mattson, 1942). It is also interesting to note that the vitamin A content continues to change throughout lactation whilst that of carotenoids does not (Chanda, 1953). In mastitis, however, the ability of the mammary gland to hold back carotenoids is so impaired that much greater amounts of β-carotene and other carotenoids find their way into the milk. The phenomenon is probably associated with a decrease in the fat content of the milk (Table 5) and a decrease in the average size of the fat micelles with a consequent increase of the area of micellar surface per unit weight of micelles (Kon & Mawson, 1950). It has been observed that the concentration of carotenoids in milk fat varies inversely with the size of the fat globules (Kon, Mawson & Thompson, 1944). The increase in the carotenoid content of mastitic-milk fat could well be due to an enhanced leakage of blood-plasma lipids into the milk through the damaged glandular epithelium, for, as is known and as Table 7 illustrates, blood serum is very much richer in carotenoids than is milk, so that it would not require much leakage to account for the difference observed in the milk of infected quarters. Vitamin A alcohol, like the carotenoids, would be expected to be more surface-active than vitamin A ester and therefore to be like the carotenoids preferentially concentrated in the chylomicrons, which have a greater ratio of surface area to mass than do normal milk globules. It can be seen from Table 5 that the amount of vitamin A alcohol/g. fat was increased by mastitis in much the same ratio as carotene and carotenoids were increased.

SUMMARY

1. Eight lactating cows were used to study the effect of mastitis on the concentration and partition of carotenoids, vitamin A and certain phosphorus compounds in milk. Mastitis was judged to be present when infection with *Streptococcus agalactiae* was detected. The increases of the chloride of the milk were taken as indicators of the intensity of the infection.

2. Phosphorus was considerably reduced and, since nitrogen increased, the ratio of nitrogen to phosphorus was greater in mastitic milk than in normal milk.

3. The ratio of nitrogen to phosphorus in the casein of the milk was increased by mastitis.

4. The concentrations of total phosphorus, ester phosphorus, lipid phosphorus, casein phosphorus of

fat and of non-fatty solids were decreased in mastitic milk. The values of the phosphorus fractions were negatively correlated with phosphatase, but inorganic phosphorus was positively correlated with phosphatase.

5. Mastitis caused a large decrease in the thiamine content of the milk and in the proportion of thiamine present in the cocarboxylase and protein-bound forms. The proportion of thiamine present in the free form was increased. There was a positive correlation between free thiamine and phosphatase, but between phosphatase and the cocarboxylase and protein-bound forms of thiamine there were negative correlations.

6. The carotenoid content of the milk fat was increased by mastitis, but the percentage of total carotenoids present in the form of β-carotene remained unchanged. The vitamin A content of the milk fat remained unchanged. The percentage of the vitamin A present in the milk in the alcohol form was increased by mastitis.

7. The blood serum of the mastitic cows was found to contain more lipid than that of the normal cows. The β-carotene content was, however, less in the mastitic than in the normal cows. Neither the vitamin A contents of the serum lipids nor the partition of vitamin A between ester and alcohol in the serum was affected.

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The Equilibrium Constants of the Fumarase and Aconitase Systems

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This paper is the first of a programme of investigations designed to obtain accurate data for the free-energy changes associated with the individual stages of the tricarboxylic acid cycle and related reactions. There is only one stage of the cycle, namely the reaction succinate → fumarate + 2H, for which data of satisfactory accuracy are available. In other cases, such as those dealt with in the present paper, the reported data are either not very accurate or they do not refer to the standard temperature of 25° now commonly used in thermodynamic measurements. There are also several stages for which no reliable data have as yet been reported.

Borsook & Schott (1931*a*) estimated the value of the equilibrium ratio L-malate/fumarate at 25° to be 3·2. This figure was based on electrometric measurements of the equilibrium potential of the succinate-fumarate system in the absence and presence of fumarase, and no great accuracy was claimed. Jacobsohn (1934) reported a ratio of 5·1 (25°, barbitone buffer of pH 6·8). Krebs, Smyth & Evans (1940) found values of 2·65 at 50°; 3·17 at 40°; 3·54 at 30° and 4·57 at 20°. Interpolation gives a value of 4·0 at 25°. Scott & Powell (1948) found a ratio of 4·05 at 25°.

All previous measurements of the equilibrium between citric, *iso*citric and *cis*-aconitic acids in the presence of aconitase refer to blood temperature (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943).

EXPERIMENTAL AND RESULTS

Determination of the equilibrium constant in the fumarase system

Dialysed heart-muscle suspension (homogenate) served as a fumarase preparation. This was prepared in a stainless-steel homogenizer from 1 part of minced sheep heart muscle and 2 parts of water and was dialysed for 3 hr. against running tap water at room temperature. In most experiments 50 ml. 0·100 M-fumarate, 20 ml. heart-muscle suspension, 3 ml. M-$NaHCO_3$ 10 drops *sec.*-octanol and water to make up 100 ml. were gassed with 5% CO_2 in N_2, and incubated at 25°. Under these conditions pH was 7·4. At intervals samples were removed for the determination of malic and fumaric acids. A control sample containing no fumarate was incubated at the same time to supply blank values for the analyses. Phosphate buffer was avoided because it interferes with the polarimetric determination of malic acid.

Polarimetric determination of L-*malic acid.* Into a 20 ml. measuring flask were pipetted, in the following sequence, 1 ml. glacial acetic acid, 4 ml. test solution, 4 ml. water, 2 ml. 1 M-sodium citrate and 29% ammonium molybdate (w/v) to the 20 ml. mark. After thorough shaking, the mixture was filtered and the clear filtrate was poured into a 2 dm. polarimeter tube. The temperature of the solution was noted; it was usually 22°. Under these conditions pure malic acid gave the following rotations: 24·0 mg. L-malic acid in the total sample of 20 ml.: $\alpha_D^{22°}$ 3·325°; $[\alpha]_D$ 1385°; 7·11 mg. L-malic acid in 20 ml.: $\alpha_D^{22°}$ 1·05°: $[\alpha]_D$ 1475° (see Krebs & Eggleston, 1943).

Manometric determination of fumaric acid and malic acid. 10 ml. of the test solution were pipetted into a test tube containing 1 ml. 20% (w/v) metaphosphoric acid. After thorough shaking and a few minutes' standing the mixture was filtered. Fumaric acid was determined by measuring the amount of H_2 required for quantitative catalytic hydrogenation, under the conditions used by Johnson (1939) for the determination of aconitic acid. The palladium catalyst of Köppen (1932)* (10 mg.) was ground up in a mortar and with 3 ml. 2% metaphosphoric acid was placed in the main compartment of a conical manometer vessel. The side arm contained 1 ml. of the test solution, the gas space H_2. The manometer vessel was provided with a valve stopper so that the gassing could be carried out in the bath, whilst the manometers were being shaken. Commercial cylinder H_2 washed with 10% pyrogallol in 10% NaOH and acid dichromate (5% potassium dichromate in 10% (w/v) H_2SO_4) was used (Johnson, 1939). The bath temperature was 40°. When equilibrium was established the contents of side arm and main compartment were mixed. The H_2 uptake was usually completed within 30–60 min.

At the end of the experiment the contents of the cups were poured on a filter and 2 ml. of the filtrates were used for the determination of malic acid with the malic decarboxylase of *Lactobacillus arabinosus* (Korkes & Ochoa, 1948; Nossal, 1952). The main compartment of the manometer vessels contained, in addition to 2 ml. filtrate, sufficient N-KOH to bring pH to approximately 5 (usually 0·3 ml.), 0·5 ml. 0·2 M-acetate buffer (pH 5·0), 0·1 ml. 0·1 M-glucose, 0·1 ml. 2% (w/v) $MnCl_2$ and water to make up 4 ml. The side arm contained 0·2 ml. of a stock suspension of *Lb. arabinosus* (20 mg. dry weight of cells per ml.) and 0·1 ml. 0·2 M-acetate buffer, pH 5·0. The gas space contained air. Bath temperature was 35°. Cells of *Lb. arabinosus* contain small quantities of fumarase and therefore decarboxylate fumaric acid as well as malic acid. This source of error is eliminated by the prior removal of fumaric acid through catalytic hydrogenation.

* Obtained from Dr R. Köppen, Norden, Ostfriesland, Germany; also obtainable from Baker Platinum Ltd., 52 High Holborn, London, W.C. 1.

As the total volume of test solutions analysed in this procedure was 1 ml. the method requires a relatively high concentration of fumarate and malate, say, above 0·02M. A few experiments were carried out at lower concentrations and in these fumarate was extracted from 10 ml. of the solution acidified with H_2SO_4, as described for aconitic acid by Johnson (1939). The extracted material was dissolved in a small volume of 2% metaphosphoric acid, usually 1·2 ml., and 1 ml. was analysed. Quantitative ethereal extraction of malic acid proved difficult and the polarimetric method was therefore used.

It should be mentioned that the palladium catalyst can be inactivated by substances present in tissue extracts. These were not encountered in dilute extracts of heart and muscle, but were present in less dilute extracts and in baker's yeast. If inhibitory substances are present, larger amounts of catalyst are required.

Results. Data from three separate experiments, carried out after the methods had been perfected, are shown in Table 1. The concentration of fumarate plus malate was 0·05M. In considering the equilibrium values the first three measurements of Exp. 3 have to be omitted because when the samples were taken equilibrium had not been established. The other eight values for the ratios L-malate/fumarate vary between 4·34 and 4·52, the average being 4·42.

In other experiments the concentration of fumarate plus malate was 0·01M, and the buffer was 0·01M-$NaHCO_3$ with 1% CO_2 in N_2. The value for the equilibrium constant was the same within the limits of error.

Determination of the equilibrium constants in the aconitase system

Sheep heart muscle was coarsely minced in a kitchen meat mincer and suspended in about 30 vol. of distilled water. After about 5 min. the tissue was centrifuged off. The washed muscle was stored in the frozen state in a deep freeze. In order to reduce blank values in the aconitase determination, arising from the dehydrogenation of unsaturated fatty acids, the frozen material was mixed with 8 vol. of ice-cold ethanol in a Waring Blendor and filtered. The insoluble material was dried in a desiccator over H_2SO_4 and then triturated with ether. It was stored in a desiccator. Part of this material (3 g.) was suspended in a total volume of 100 ml. incubated solution. In an alternative enzyme preparation the ethanol was replaced by ice-cold acetone. Both preparations were about equally active. In all experiments the substrate was 0·200M-sodium citrate. $NaHCO_3$ (0·03M) and CO_2 (5%) served as buffer.

Aconitic acid was determined manometrically by quantitative catalytic hydrogenation (Johnson, 1939). 8 ml. of the incubated materials were deproteinized with 2 ml. 20% (w/v) metaphosphoric acid and a measured amount of the filtrate (7–8 ml.) was extracted with ether in a continuous extractor. Extraction was found to be completed within 2 hr. The ether-soluble material was collected in a total volume of 1·5 ml. 2% metaphosphoric acid and 1 ml. of this solution was placed in the side arm of a manometer vessel. Otherwise the procedure was as described for the determination of fumaric acid. *iso*Citric acid was determined polarimetrically under the conditions described by Eggleston & Krebs (1949).

Results. The results shown in Table 2 are reasonably consistent. A possible source of error is the gradual formation of *trans*-aconitic from *cis*-aconitic acid. The fact that analytical results after 6 and 24 hr. showed no change indicates that no significant amounts of *trans*-aconitic acid were formed under the test conditions.

The average value for the *iso*citric acid formed was 178 μl./ml. and of *cis*-aconitic acid 130 μl./ml. The most reliable determination of citric acid is by difference and the equilibrium mixture at 25° thus contained 6·20% *iso*citric acid, 2·90% *cis*-aconitic acid and 90·9% citric acid. The corresponding figures at 38° are 6·6% *iso*citric acid, 4·3% *cis*-aconitic acid, 89·1% citric acid (Krebs & Eggleston, 1943; Eggleston & Krebs, 1949).

Table 1. *Equilibrium concentrations of fumarate and malate in the presence of fumarase at* pH 7·4 (25°)

(For procedure see text. The manometric results of the malate determinations were used for the calculation of the malate/fumarate ratio. In Exp. 3 a dialysed fumarase solution which had been stored about 6 days in the refrigerator and which was less active was used.)

Exp. no.	Period of incubation (hr.)	L-Malate: Manometrically (μl./ml.)	L-Malate: Polarimetrically (μl./ml.)	Fumarate (μl./ml.)	Ratio: L-malate / fumarate
1	2	900	—	207	4·34
	4	886	—	201	4·41
	5	884	—	202	4·37
	7	910	—	201	4·52
2	2·5	902	924	205	4·42
	5	904	905	204	4·43
	24	920	922	204	4·52
3	2	791	778	310	2·55
	4	843	852	250	3·37
	6	894	902	210	4·29
		909	919	207	4·38

Table 2. *Equilibrium concentrations of* iso*citrate and* cis*-aconitate in the presence of aconitase at* pH 7·4

(For procedure see text. Temp., 25°. All data corrected for blank. Total tricarboxylic acid concentration 0·200 M (4480 μl./ml.).)

Exp. no.	Enzyme preparation	Period of incubation (hr.)	*iso*Citrate $\alpha_D^{22°}$	*iso*Citrate (μl./ml.)	*iso*Citrate % of total tricarboxylic acid	*cis*-Aconitic acid (μl./ml.)	*cis*-Aconitic acid % of total tricarboxylic acid
1	Ethanol	24	−1·69	276	6·16	133	2·97
						128	2·86
						126	2·81
						133	2·97
2	Ethanol	18	−1·71	280	6·26	129	2·88
						130	2·90
						133	2·97
						135	3·01
3	Ethanol	6	−1·72	281	6·21	129	2·58
		24	−1·70	278	6·21	131	2·93
4	Acetone	6	−1·60	262	—	122	—
		24	−1·70	278	6·21	127	2·84
5	Ethanol	24	−1·70	278	6·21	—	—
6	Washed sheep heart	6	−1·70	278	6·21	127	2·84
		24	−1·69	276	6·16	129	2·88
					Av. 6·20		2·90

NOTE ON THE EFFECT OF THE HYDROGEN-ION CONCENTRATION ON THE EQUILIBRIUM RATIOS

If, at a given temperature, the equilibrium ratio for one of the three forms in which the two dibasic acids occur in aqueous solutions (undissociated, univalent anion, divalent anion), and the ionization constants of the acids are known, the value of the equilibrium ratio L-malic acid (all forms)/fumaric acid (all forms) can be calculated for any hydrogen-ion concentration.

Let H_2A, HA^- and A^{2-} be the undissociated form and the uni- and bi-valent anions respectively of a dibasic acid and T the sum of H_2A, HA^- and A^{2-}. It follows from the equations defining the ionization constants that

$$[A^{2-}]=\frac{[T]\,K_1K_2}{K_1K_2+[H^+]^2+[H^+]\,K_1}, \qquad (1)$$

where $[H^+]$ is the H-ion concentration and K_1 and K_2 are the two ionization constants (see Michaelis, 1922). Hence,

$$\frac{[A^{M2-}]}{[A^{F2-}]}=\frac{[T^M]}{[T^F]}\times\frac{K_1^MK_2^M[K_1^FK_2^F+[H^+]^2+[H^+]K_1^F]}{K_1^FK_2^F[K_1^MK_2^M+[H^+]^2+[H^+]K_1^M]}, \qquad (2)$$

where the superscripts M and F refer to malic and fumaric acids. At pH 7·4 $[A^{M2-}]/[A^{F2-}]$ is practically the same as $[T^M]/[T^F]$, i.e. equal to the ratio measured in the experiments recorded in Table 1, henceforth referred to as r. Substituting r for $[A^{M2-}]/[A^{F2-}]$ in (2):

$$\frac{[T^M]}{[T^F]}=r\times\frac{K_1^FK_2^F[K_1^MK_2^M+[H^+]^2+[H^+]K_1^M]}{K_1^MK_2^M[K_1^FK_2^F+[H^+]^2+[H^+]K_1^F]}. \qquad (3)$$

This formula may be used to calculate the equilibrium concentrations of total malic and fumaric acids at any H-ion concentration. If $[H^+]$ is small compared with K_1 and K_2 (below 10^{-7}) all terms containing $[H^+]$ become negligible and $[T^M]/[T^F]=r$. As $[H^+]$ rises the numerator of the fraction increases more rapidly than the denominator because K_1^F and K_2^F are greater than K_1^M and K_2^M. $[T^M]/[T^F]$ rises towards a maximum value of $r\times K_1^FK_2^F/K_1^MK_2^M$. Assuming $r^{25°}=4·42$ and

$K_1^F=9·6\times10^{-4}$; $K_2^F=4·0\times10^{-5}$ (German & Vogel, 1936; Sihvonen, 1930),

$K_1^M=3·3\times10^{-4}$; $K_2^M=7·7\times10^{-6}$ (Clark, 1928),

the following values for $[T^M]/[T^F]$ are obtained

pH	$[T^M]/[T^F]$
7·4	4·42
7·0	4·46
6·0	4·86
5·0	8·26
4·0	22·4
3·0	44·6
2·0	62·0
1·0	66·4

A graphical presentation of the effect of pH on the equilibrium ratio is given in Fig. 1.

To test the theory the equilibrium was measured at pH 4·85. As fumarase loses its activity at lower pH ranges the test cannot be extended over the whole range. Owing to the weak activity of the enzyme at pH 4·85 relatively large amounts of fumarase have to be used. Sheep heart was minced in the Latapie mill and stored at −12°. To prepare an extract the frozen material was allowed to thaw and homogenized in the Waring Blendor with 2 vol. of water. The homogenate was centrifuged and the supernatant, after 2 hr. dialysis against running tap water, served as a source of fumarase. 20 ml. extract, 1 ml. 0·5 M-fumarate, 2·5 ml. M-acetate buffer, pH 4·7, 1·5 ml. water and a few drops *sec.*-octanol were incubated at 25°, together with a control containing no fumarate. At intervals 5 ml. samples were removed and mixed immediately with 1 ml. 40% (w/v) metaphosphoric acid. The filtrate was used for the determination of fumarate as described before except that 50 mg.

catalyst instead of 10 mg. were used. This modification proved necessary because the tissue extract contained substances which reduced the activity of the catalyst. pH was measured at the end of the incubation period; the value of 4·85 was somewhat higher than that of the buffer owing to the neutralizing effects of substrate and enzyme solution.

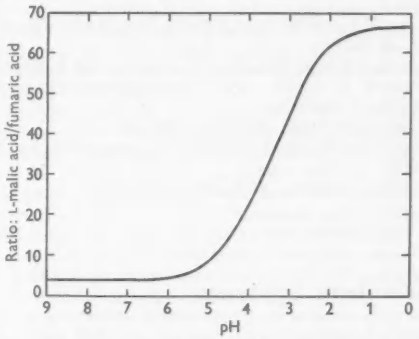

Fig. 1. Effect of pH on the equilibrium ratio malic acid (all forms)/fumaric acid (all forms) calculated according to eqn (3).

The results were as follows:

	4 hr.	6 hr.
L-Malate (μl./ml.)	398	404
Fumarate (μl./ml.)	49	50

The ratios malate/fumarate were thus 8·1. This is somewhat lower than calculated, but the deviation is not unexpected as the ionization constants used for Fig. 1 are the thermodynamic ionization constants. At the ionic strength of the incubated solutions the ionization constants would be greater which would result in a shift of the S-shaped curve of Fig. 1 to the right.

The theoretical treatment of this problem is analogous to that of the effect of pH on oxido-reduction potentials (Borsook & Schott, 1931*b*). Corresponding equations can be derived for mono- or tri-carboxylic acids. In the case of a monobasic acid (1) becomes

$$[A^-] = \frac{[T]K}{[H^+]+K},$$

and (3)
$$\frac{[T_I]}{[T_{II}]} = \frac{[A_I^-]}{[A_{II}]} \times \frac{K^{II}([H^+]+K^I)}{K^I([H^+]+K^{II})}. \qquad (4)$$

If the ionization constants of di- and poly-basic acids are very different, such acids may be treated, within a limited pH range, as monobasic acids. This applies, for example, to the equilibria in various phosphatase systems, where it is sufficient, within the pH range of enzyme activity, say pH 5–9, to consider only the second ionization constant.

Examples illustrating the validity of equation (4) are provided by data published in papers of Cori, Cori & Green (1943) and of Hanes (1940*a*, *b*). In the phosphorylase system

glycogen + phosphate ⇌ glucose 1-phosphate

the equilibrium ratio total inorganic phosphate/total glucose phosphate was found to vary between 2·45 and 10 when pH varied between 5 and 7·3,

Table 3. *Equilibrium ratio total inorganic* P/*total glucose* 1-P *in the presence of phosphorylase*

(The observed ratios in series 1 are taken from Cori *et al.* (1943), Table 3, in series 2 from Hanes (1940*b*) Table 7. For the calculation according to equation (4) the equilibrium ratio of the bivalent anions was assumed to be 2·0 (Cori *et al.* 1943); K_2 of inorganic phosphate = $1·51 \times 10^{-7}$; K_2 of glucose 1-phosphate = $7·76 \times 10^{-7}$ (Cori, Colowick & Cori, 1937.)

Series	$[H^+]$ (M)	Observed ratio	Calculated ratio
1	$0·47 \times 10^{-7}$	2·45	2·48
	$0·10 \times 10^{-7}$	3·11	3·03
	$3·09 \times 10^{-7}$	4·08	4·36
	$8·51 \times 10^{-7}$	5·90; 6·81	6·44
2	1×10^{-7}	3·1	2·9
	$3·2 \times 10^{-7}$	4·7	4·4
	10×10^{-7}	6·7	6·7
	32×10^{-7}	9·0	8·4
	100×10^{-7}	10·8	9·7

whilst the ratios of the bivalent and univalent anions were constant. Table 3 shows that the above ratio can be satisfactorily calculated for different pH values if the ratio for the bivalent anions is known.

SUMMARY

1. The concentrations of fumarate and L-malate, and of *iso*citrate, aconitate and citrate have been measured in the aqueous equilibrium mixtures in the presence of fumarase and aconitase.

2. At equilibrium, the fumarase system was found to contain 18·45 % fumarate and 81·55 % L-malate, the aconitase system 6·20 % *iso*citrate, 2·90 % *cis*-aconitate and 90·9 % citrate (pH 7·4; 25°).

3. A theory is given for the calculation of the effect of pH on the equilibrium concentrations of systems containing acids or bases. The experimental test of the theory in the fumarase and phosphorylase systems proved satisfactory.

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Equilibria in Transamination Systems

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Preliminary determinations of the equilibrium constants in the glutamic-aspartic and the glutamic-alanine system by Cohen (1940) gave the following values at 38°:

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-aspartate}]}{[\text{L-glutamate}] \times [\text{oxaloacetate}]} = 3{\cdot}5, \qquad (1)$$

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-alanine}]}{[\text{L-glutamate}] \times [\text{pyruvate}]} = 1. \qquad (2)$$

Improved techniques employed by Darling (1945, 1947) gave 5·44 for (1) and 2·25 for (2) whilst Lénárd & Straub (1942) reported a value of 1·43 for (2). In the present work the two constants were redetermined with the help of the newer more accurate manometric methods for the determinations of glutamic, α-ketoglutaric, aspartic, pyruvic and oxaloacetic acids. Under optimum conditions the probable error of a single estimation of any of these substances is not greater than 1%, but a complication arises in the determination of the equilibrium constant of (1) from the instability of oxaloacetate; its slow conversion into pyruvate and bicarbonate is unavoidable and no perfect equilibrium can be reached. However, any error due to this side reaction is negligible if the rate of the side reaction is slow in relation to that of transamination. The presence of additional substances formed by side reactions does not cause errors if the concentrations of all four reactants in the transamination systems are determined directly.

EXPERIMENTAL AND RESULTS

Measurements in the glutamic-aspartic system

Enzyme preparation. Finely minced horse heart muscle was extracted with 2 vol. 0·5% NaCl solution for 60 min. and then centrifuged. The supernatant was dialysed for at least 4 hr. against running tap water to remove preformed substrates. The dialysed extract was stored in the refrigerator after addition of, and thorough mixing with, *sec.*-octanol. The extract contained a powerful glutamic-aspartic transaminase and showed weak alanine-glutamic transaminase and oxaloacetic decarboxylase activity. That the rate of side reactions under the test conditions was very small can be judged from data given in Table 1. In the absence of the side reactions the sum of aspartic and glutamic acids should remain constant and should equal the amount of glutamate added, any deficit being due to the formation of alanine. Furthermore, the sum of oxaloacetate and aspartate should be constant and equal the amount of aspartate added, any loss being due to the decarboxylation of oxaloacetate.

Reaction mixture and sampling for analysis. Equal volumes (10–15 ml.) of about 0·08 M-α-ketoglutarate, about 0·08 M-L-aspartate, enzyme solution and 0·1 M-phosphate buffer, pH 7·4, and a few drops of *sec.*-octanol were mixed and placed in a water bath at 25·0°. The actual initial concentrations of the α-ketoglutarate and L-aspartate solutions were determined in each experiment. A control mixture contained buffer and enzyme but no substrate. At specified times samples were removed for analysis. For the determination of oxaloacetic acid 3 ml. were placed in the main compartment of an ice-cooled conical manometer vessel containing 1 ml. 2 N-HCl. For the determination of the three other reactants 9 ml. were pipetted into a graduated

test tube containing 1 ml. 2N-NaOH, to stop the enzyme activity; these samples were then placed for 30 min. in a boiling-water bath to inactivate the enzyme and to decompose the oxaloacetate. Care was taken to avoid loss of water by evaporation. Under these conditions there was no loss of glutamate, aspartate or α-ketoglutarate, but losses of glutamic acid up to 10% occurred when NaOH was replaced by HCl. After cooling, glutamate was determined in 3 ml., α-ketoglutarate and aspartate in 0·5 ml. All analyses were carried out in duplicate and specially calibrated pipettes were used. The amounts of gas measured were of the order of 300 μl. in all four cases.

Analytical procedures. Oxaloacetate was determined manometrically according to Ostern (1933). The side arm of the vessel containing the acidified solution of oxaloacetic acid was charged with 0·5 ml. aniline. HCl was used for acidification rather than citric or acetic acids because the spontaneous decomposition of oxaloacetic acid is slower in strongly acid medium. The manometric determination was carried out at 3–5° in a cold room. Under these conditions loss owing to the spontaneous decomposition of oxaloacetic in the equilibration period was negligible. The solubility of CO_2 in the reaction mixture was found to be the same as in pure water. This was determined by adding a known amount of bicarbonate to the solution and using the equation for the vessel constant for the calculation of α_{CO_2}.

Glutamic acid was determined with the specific decarboxylase of *Clostridium welchii* S.R. 12 (Krebs, 1948, 1950). The 3 ml. sample of the alkaline solution was neutralized in the manometer vessel by 0·3 ml. 2N-HCl. In addition, 0·5 ml. M-acetate buffer, pH 4·5, 0·5 ml. 2% Cetavlon (cetyltrimethylammonium bromide) and 0·05 ml. aniline were added. Under these conditions the presence of aspartic and pyruvic acids in the test solution did not interfere with the determination of glutamic acid. Meister, Sober & Tice (1951) have found that suspensions of *Cl. welchii* can form CO_2 and α-alanine in the presence of aspartic acid provided that small (catalytic) quantities of an α-ketonic acid are present. This reaction may interfere with the determination of glutamic acid under some conditions, but it does not occur in the presence of Cetavlon. The bacterial suspension did not decarboxylate pyruvic acid (see Boulanger & Osteux, 1949; Krebs, Boulanger & Osteux, 1950).

L-Aspartate and α-ketoglutarate were determined in 0·5 ml. samples of the alkaline solutions according to the method of Krebs (1950). Special care was taken to ensure the quantitative liberation of CO_2, by adjusting the pH of the solutions to a value below 5·0. They were brought to approximately this pH with acid or alkali before the addition of Cetavlon, bromocresol green serving as external indicator, and 0·5 ml. 0·2 M-acetate buffer of pH 4·5 was added to the main compartment, 0·2 ml. to the side arm.

Results. Preliminary experiments showed that under the conditions of the experiments equilibrium was established within about 20 min. In the main experiments, the solutions were analysed after 30, 40 and 50 min. incubation. The results are given in Table 1. As will be seen the value of the equilibrium constant K remained virtually constant during incubation although, owing to the two side reactions mentioned, the concentration of the reactants changed slightly. The concentrations of oxaloacetate, aspartate and α-ketoglutarate fell, that of glutamate rose very slightly. The eighteen recorded values of K vary between 6·45 and 7·06, the mean being 6·74. Darling (1945) gives four measurements in support of his average value of 5·44, the range being 4·1–6·3. Only oxaloacetate was determined (colorimetrically) and the concentration of the other components was calculated from their initial quantity and the oxaloacetate value, a procedure which is likely to be less reliable than that used in the present paper.

Table 1. *Equilibrium in the aspartic-glutamic-transaminase system*

(Temp., 25°; pH 7·4; concentrations are expressed in μl. substrate/ml. solution.)

Exp. no.	Period of incubation (min.)	Concentrations found				K
		Oxaloacetate	Glutamate	α-Ketoglutarate	Aspartate	
1	30	111	121	298	307	6·81
		111	122	297	306	6·71
	40	106	120	289	298	6·78
		104	123	289	284	6·78
	50	108	123	297	305	6·82
		100	123	292	297	7·06
2	30	114	122	315	300	6·78
		115	122	316	302	6·82
	40	111	122	313	294	6·78
		111	124	313	293	6·68
	50	108	127	319	296	6·72
		109	127	314	289	6·45
3	30	119	123·5	324	309	6·82
		118	123	324	314	7·01
	40	117	124	318	302	6·62
		113	123	315	306	6·65
	50	115	127·5	311	300	6·60
		114	126	315	298	6·48

Measurements in the glutamic-alanine system

Enzyme preparation. Sheep heart, removed from the animal within a few minutes after death, was carried in ice from the abattoir to the laboratory and on arrival the muscle tissue, freed from fat, was minced in the Latapie mill. 1 part of mince was stirred into 2 parts of 0·9% NaCl solution. After standing at room temperature for 4 hr., the suspension was filtered through muslin, and to each 100 ml. filtrate 35 g. $(NH_4)_2SO_4$ were added. The precipitate containing the enzyme was allowed to form overnight in the refrigerator and was centrifuged off; the inactive supernanant was discarded. The precipitate was stirred into a small volume of water to form a liquid paste which was dialysed for 16 hr. against running tap water. Insoluble matter in the dialysed material which proved inactive was separated by centrifugation. The active supernatant was dried *in vacuo* over H_2SO_4. About 1 g. dry powder was obtained from 1 kg. fresh heart muscle. It was pink coloured and readily soluble in water. To test the activity, 0·25 ml. 0·5 M-phosphate buffer, pH 7·4, 0·25 ml. 0·4 M-sodium pyruvate, 0·25 ml. 0·4 M-sodium glutamate and 4·25 ml. enzyme solution were incubated at 25°. After 30 min. the reaction was stopped by the addition of 0·5 ml. 2 N-HCl and heating to 100° for 10 min. α-Ketoglutarate was determined manometrically in 1 ml. of this solution; 1 mg. of the dry powder produced about 1·5 mg. α-ketoglutaric acid under these conditions.

As precipitation with $(NH_4)_2SO_4$ always caused considerable losses (see O'Kane & Gunsalus, 1947) a concentrated, dialysed tissue extract was used in some experiments. The tissue extract prepared from minced tissue as described above was reduced *in vacuo* to about one-fifth of the original volume and the concentrate was dialysed against running tap water. The solution obtained after the removal of insoluble particles by centrifugation was stored in the frozen state. Horse heart gave less active enzyme preparations. Heating of the tissue suspension to 55° for 50 min. (see Lénárd & Straub, 1942) did not increase the yield.

Reaction mixture and sampling. 2 ml. 0·4 M-L-glutamate (or α-ketoglutarate), 2 ml. 0·4 M-pyruvate (or 0·8 M-DL-alanine), 4 ml. 0·5 M-phosphate buffer, pH 7·4 and 32 ml. enzyme solution were incubated at 25°. Controls contained buffer and enzyme, but no substrates. Three types of special controls containing either all constituents except glutamate, or all constituents except pyruvate, or all constituents except the enzyme were set up in the first experiments to test the disappearance of substrates by reactions other than

Table 2. *Equilibrium in the glutamate-alanine-transaminase system*

(Initial substrates: L-glutamate and pyruvate. Temp., 25°, pH 7·4. Concentrations expressed as μl. substrate/ml. solution. Enzyme preparation 1:20 ml. dialysed heart extract in total volume of 40 ml.; enzyme preparation 2:70 mg. dry powder in total volume of 40 ml.)

Exp. no.	Period of incubation (hr.)	Enzyme preparation	Initial substrate concentration		Final substrate concentration			Equilibrium constant $\frac{[\alpha\text{-Ketoglutarate}]\times[\text{L-alanine}]}{[\text{L-glutamate}]\times[\text{pyruvate}]}$
			L-Glutamate	Pyruvate	L-Glutamate	α-Ketoglutarate and alanine	Pyruvate	
1	4	1	432	428	196	235	193	1·48
	6				197	238	190	1·51
	24				197	238	190	1·51
					197	238	190	1·51
					198	231	197	1·37
2	3	1	426	431	196	236	195	1·46
					196	235	196	1·44
	4				200	232	199	1·38
					200	236	200	1·39
	6				194	239	192	1·54
					196	235	196	1·44
	24				191	230	201	1·38
					193	229	202	1·38
3	6	2	432	433	193	239	194	1·53
					193	241	192	1·57
	24				193	241	192	1·57
					191	241	192	1·58
4	3	2	454	470	194	252	208	1·57
					196	250	210	1·52
	4				195	251	209	1·43
					196	250	210	1·52
	6				194	252	208	1·47
					194	252	208	1·47
	24				191	255	205	1·66
					194	252	208	1·57
							Av.	1·50

Table 3. *Equilibrium in the glutamate-alanine-transaminase system*

(Initial substrates: α-ketoglutarate and DL-alanine. For other particulars see Table 2.)

Exp. no.	Period of incubation (hr.)	Enzyme preparation	Initial substrate concentrations: α-Ketoglutarate	Initial substrate concentrations: DL-Alanine	Final substrate concentrations: L-Glutamate and pyruvate	Final substrate concentrations: α-Ketoglutarate	Final substrate concentrations: L-Alanine	Equilibrium constant: [α-Ketoglutarate] × [L-alanine] / [L-glutamate] × [pyruvate]
1	3	1	463	896	203	257	245	1·53
					205	256	243	1·48
	4				201	250	249	1·54
					200	252	248	1·56
	6				201	246	247	1·50
					202	245	246	1·47
	24				201	246	247	1·50
					200	247	248	1·51
2	3	2	461	496	197	259	251	1·67
					198	260	250	1·66
	4				202	261	246	1·57
					202	260	246	1·57
	6				193	258	249	1·62
					203	256	245	1·52
	24				201	257	247	1·57
					200	259	248	1·61
							Av.	1·56

transamination. These tests were negative. The sample for the determination of glutamic acid was treated with alkali and that for the determination of α-ketoglutarate with acid as described above.

Analytical procedure. The procedures for the determination of glutamate and α-ketoglutarate have already been described. In addition the initial concentrations of the reactants was known. The concentration of pyruvate in the stock solutions (from which initial pyruvate concentration in the reaction mixture was obtained) was determined by the carboxylase method and the ceric sulphate methods (Krebs & Johnson, 1937) which gave identical results with pure solutions. In the carboxylase method the pH of the acetate buffer was 4·5 to ensure that the CO_2 evolution was quantitative, 0·02 vol. 3M buffer being used to acidify the yeast extract and 0·5 ml. 0·2M buffer to acidify the solution in the main compartment. The direct determination of pyruvate in the equilibrium mixture was not attempted because α-ketoglutarate interfered with the method of analysis.

Reagents. As the concentrations of alanine and pyruvate were determined by difference, the purity of these substances was of importance. Solutions of aqueous pyruvic acid or sodium pyruvate were found to change on storage in the refrigerator, forming substances (presumably condensation products) which were decarboxylated by yeast carboxylase but did not react in the transaminase system. Pure pyruvic acid solutions were obtained as follows. Freshly prepared pyruvic acid was subjected to fractional distillation *in vacuo* and weighed portions of the middle fractions were immediately diluted with about 4 vol. of water. The strength of this stock solution was determined by the carboxylase and ceric sulphate methods and was found to agree with the calculated value. On storage of frozen solutions at $-10°$ no changes occurred within 3 months. For use, a sample of stock solution was freshly neutralized with an equivalent amount of $NaHCO_3$ immediately before the start of the experiment. Commercial L-alanine (Roche Products Ltd.) was found by paper chromatography to contain an impurity (serine or threonine) and also traces of D-amino-acid when tested by D-amino-acid oxidase. DL-Alanine was therefore used. Its assay with D-amino-acid oxidase showed that its purity was above 99·5%. D-Alanine did not react with the transaminase preparation used.

In all experiments, irrespective of whether glutamate, pyruvate or α-ketoglutarate and alanine were the substrates, both α-ketoglutarate and glutamate were determined in the incubated solution. When L-glutamate and pyruvate were the substrates, the equilibrium concentration of L-alanine was taken to be the same as that of α-ketoglutarate and that of pyruvate to be the difference between its initial concentration and the final concentration of α-ketoglutarate. When α-ketoglutarate and DL-alanine were the substrates, the equilibrium concentration of pyruvate was taken to be the same as that of glutamate and that of L-alanine to be the difference between its initial concentration and that of the equilibrium concentration of glutamate. The assumptions made would become invalid if side reactions occurred, but there is no evidence suggesting that this was the case. The sum of the two C_5 compounds recovered agreed with the amounts added, and once equilibrium was established the concentrations of the two C_5 compounds remained constant (Tables 2 and 3). These findings ruled out significant side reactions of the two C_5 compounds.

Results. The results of the measurements carried out after the perfection of the methods are given in Tables 2 and 3. The same equilibrium position was reached from both sides, the average value for the constant being 1·52. This is in fair agreement with the value of 1·43 of Lénárd & Straub (1942).

SUMMARY

The equilibrium constants of two transamination systems have been measured at 25°. The equilibrium constants, as defined in equations (1) and (2) were found to be, on the average, 6·74 in the glutamic-aspartic system and 1·52 in glutamic-alanine system.

The author wishes to acknowledge the technical assistance of Mrs Edith Skipworth.

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The Free-energy Changes for the Reduction of Diphosphopyridine Nucleotide and the Dehydrogenation of L-Malate and L-Glycerol 1-Phosphate

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The free energy of reduction of diphosphopyridine nucleotide (DPN) may be evaluated by adding the free-energy change calculated from the equilibrium of a suitable DPN dehydrogenase system (1) to the free-energy change for the reduction of the substrate (2)

$$AH_2 + DPN^+ \rightarrow A + DPNH + H^+, \quad (1)$$

$$A + H_2 \rightarrow AH_2, \quad (2)$$

$$DPN^+ + H_2 \rightarrow DPNH + H^+. \quad (3)$$

The standard free-energy change (ΔG^0) of reaction (3) is related to the oxidation-reduction potential (E_0')

$$E_0' = -\frac{\Delta G^0}{2F} - \frac{RT\,\mathrm{pH}}{2F \log e},$$

where F is the Faraday (23068 cal.), R the gas constant and T the absolute temperature. By this procedure, several workers have obtained values for the oxidation-reduction potential of DPN using data for different hydrogenase systems (Green & Dewan, 1937; Clark, 1938; Schlenk, Hellström & Euler, 1938; Borsook, 1940). Several serious discrepancies appear when the available free-energy data for different dehydrogenase systems are critically examined, e.g. the generally accepted value for the DPN potential ($-0{\cdot}282$ V. at pH 7 and 30°; Borsook, 1940) differs from that ($-0{\cdot}320$ V. at pH 7 and 25°) which may be obtained from equilibrium and thermochemical data for the ethanol-DPN-acetaldehyde system (Dixon, 1949; Burton, 1952). A value closer to that of Borsook (namely $-0{\cdot}29$ V. at pH 7 and 25°) is indicated by the potential measurements of Barron & Hastings (1934) and the equilibrium data of Kubowitz & Ott (1943) and Racker (1950) for the lactic dehydrogenase system. Another value of about $-0{\cdot}21$ V. may be obtained by combining the potential measurements of Lehmann & Hoff-Jörgensen (1939) for the malate-oxaloacetate reaction with equilibrium data given by Schlenk *et al.* (1938).

The object of the work described in this paper was, first, to obtain a reliable independent value for the free-energy change (ΔG^0) for the reduction of DPN (i.e. the oxidation-reduction potential), and secondly to use this value in conjunction with equilibrium data to obtain the free-energy change of several biological dehydrogenations. To evaluate ΔG^0 for the reduction of DPN, the equilibrium constant has been measured for the reaction:

*iso*propanol + DPN^+ → acetone + DPNH + H^+, (4)

which is catalysed by crystalline preparations of yeast alcohol dehydrogenase. This reaction was

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investigated because accurate free-energy data are available for both acetone and *iso*propanol from thermochemical measurements. The value obtained for ΔG^0 of reduction of DPN from these data is in good agreement with that similarly obtained using published equilibrium and thermochemical data for the ethanol-acetaldehyde system. Equilibrium measurements are also reported for the malic and glycerol 1-phosphate dehydrogenase systems.

As will be shown in a later paper (Burton & Krebs, 1953), the data obtained here may be combined with other data to give comprehensive free-energy data for glycolysis and the citric acid cycle.

The value for the oxidation-reduction potential of DPN has been briefly reported elsewhere (Burton, 1952).

THERMODYNAMIC SYMBOLS AND CONVENTIONS

The conventions adopted are those of Lewis & Randall (1923) and the symbols are defined in the following paper with the exception of E'_0. This is the equilibrium e.m.f. of a cell consisting of an inert electrode in the aqueous oxidation-reduction system at a given pH and a hydrogen electrode (hydrogen at 1 atm.) in the same solution: all relevant components except acid are at unit activity. An account of the theory of oxidation-reduction potentials is given by Johnson (1949).

The unit of energy used in the text is the kilocalorie (kcal.), but some of the more important values (Table 3) are given both in kcal. and in kilojoules (kJ.). 1 calorie = 4·1840 absolute joules.

EXPERIMENTAL AND RESULTS

Diphosphopyridine nucleotide (DPN). This was obtained from local brewer's yeast by a method similar to that of LePage (1949), developed in co-operation with Mr D. E. Hughes. The stages after preparation of the crude extracts were performed in a different order, namely (*a*) charcoal adsorption and elution, (*b*) treatment with lead acetate, (*c*) precipitation and subsequent decomposition of the silver salt, and (*d*) precipitation by acidified acetone. The final products of several preparations were 70–85% pure. Yield 0·05 g./kg. yeast. The material used for the experiments described in this paper was 74% pure (calculated on the basis of the molecular formula $C_{21}H_{27}O_{14}N_7P_2$, mol.wt. 663). DPN was estimated spectrophotometrically after enzymic reduction by ethanol (Racker, 1950); the molecular extinction coefficient of reduced DPN at 340 mμ. was taken as $6{\cdot}22 \times 10^3$ cm.^{2}/mole (Horecker & Kornberg, 1948).

Enzymes. Highly purified preparations of malic dehydrogenase (Straub, 1942) and diaphorase (Straub, 1939) were from horse heart muscle; crystalline alcohol dehydrogenase (Racker, 1950) was from 'Premier' baker's yeast. A crude preparation of glycerol 1-phosphate dehydrogenase was obtained by fractionating an aqueous extract of guinea-pig skeletal muscle between 30 and 55% saturation with $(NH_4)_2SO_4$ (Racker, 1947). Before use all enzyme preparations were dialysed with stirring against 0·05 M-phosphate buffer, pH 7·4, to remove $(NH_4)_2SO_4$.

iso*Propanol.* This was purified according to Gilson (1932).

pH. This was measured at 25° using a glass electrode, 3·5 M-KCl bridge and 3·5 N-calomel electrode. The system was calibrated according to British Standard 1647: 1950.

Spectrophotometric measurements. These were made with a Beckman model DU spectrophotometer fitted with a constant temperature chamber (cf. Gibson & Balcom, 1947).

Enzymic dehydrogenation of isopropanol

The decolorization of methylene blue in the presence of *iso*propanol and a crude preparation of yeast alcohol dehydrogenase was described by Müller (1934), but the product of the reaction was not identified and the requirement for coenzyme not examined.

The reaction has been further investigated using crystalline yeast alcohol dehydrogenase. In the presence of excess *iso*propanol DPN was completely reduced as indicated by the light absorption at 340 mμ. On subsequent addition of acetone the reduced DPN could be reoxidized. These observations suggested that yeast alcohol dehydrogenase can catalyse reaction (4) in addition to the analogous reaction between ethanol and acetaldehyde. About 500 times as much enzyme is required to obtain the same rate of reaction with 0·03 M-*iso*propanol as that obtained with the same concentration of ethanol (pH [illegible] 10^{-5} M-DPN).

To confirm that acetone is the product of the reaction, it was isolated as its 2:4-dinitrophenylhydrazine derivative. A solution containing alcohol dehydrogenase (9×10^6 units; Racker, 1950), 70 mg. *iso*propanol, 8 mg. methylene blue, diaphorase (equivalent to 3×10^{-11} moles riboflavin) and 40 mg. 50% pure DPN in a total volume of 20 ml. was oxygenated by shaking in a 100 ml. reaction vessel fitted to a Warburg manometer (Krebs & Eggleston, 1945). After 6 hr., the rate of O_2 consumption became very slow. On the assumption that the O_2 was reduced to H_2O_2, the amount consumed (3·8 ml.) corresponded to 15% conversion of *iso*propanol to acetone. The enzymes were then inactivated by adding 0·8 ml. 2 N-NaOH and the solution was distilled for 15 min. into a receiving flask containing 10 ml. 2 N-HCl saturated with 2:4-dinitrophenylhydrazine. A copious yellow crystalline precipitate was immediately formed. No precipitate was formed in a control experiment where the *iso*propanol was added after inactivation of the enzymes by alkali. The material was twice recrystallized from aqueous ethanol and identified as acetone 2:4-dinitrophenylhydrazone by its m.p. (126° corr.) and the mixed m.p. (125°) with an authentic sample which itself melted at 125°. Values for the m.p. of this compound given in the literature are 122–124° (Dirscherl & Nahm, 1940) and 125–126·5° (Roberts & Green, 1946); it is not stated whether these values were corrected for the emergent stem.

Although it is feasible that reaction (4) is catalysed not by the enzyme which acts on ethanol but by an impurity in the enzyme preparation, this is considered to be unlikely since the ratio of the rates towards *iso*propanol and ethanol were similar at different stages of purification.

Equilibrium measurements. Most measurements were performed in pairs, each with the same concentration of constituents but added in different orders so that the equilibrium was approached from opposite sides (Fig. 1).

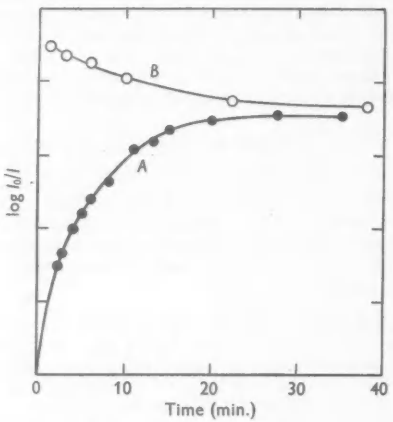

Fig. 1. Equilibrium of the *iso*propanol-DPN-acetone reaction approached from both sides. See Table 1. Temp., 25°; pH 7·64; $5·5 \times 10^{-3}$ M-pyrophosphate; 0·0195 M-acetone; 0·0978 M-*iso*propanol; 10^{-4} M-DPN.

In the first measurement of each pair (*A*) crystalline alcohol dehydrogenase was added to a solution containing acetone, *iso*propanol, and DPN. The reduction of the DPN was followed spectrophotometrically and allowed to proceed to equilibrium. In the second measurement (*B*), the enzyme was added to the same solution without acetone. The reduction of DPN by *iso*propanol was allowed to proceed beyond the equilibrium value obtained in (*A*). Acetone was then added and the reduced DPN became reoxidized until equilibrium was attained.

The equilibrium concentrations of acetone and *iso*propanol were taken as the initial values since the proportion reacting was in each case less than about 0·2%. Although values may be obtained for the concentrations of reduced and oxidized DPN at equilibrium from the initial concentrations and the spectrophotometer readings, the following procedure was adopted to ensure that no side reaction, such as destruction of the nucleotide, had occurred to any appreciable extent. The equilibrium concentration of reduced DPN was determined by adding 0·01 ml. of 10% acetaldehyde to 1·5 ml. of the equilibrium mixture. The optical density rapidly decreased to a value very close to that of the original solution of DPN, buffer, *iso*propanol and acetone. This fall in optical density was used to calculate the reduced DPN present at equilibrium. The oxidized DPN was determined from the increase of optical density when 2·0 ml. of the equilibrium mixture was mixed with 0·5 ml. of a solution containing 0·25 M-$Na_4P_2O_7$ and 0·5 M-ethanol. Under these conditions all the DPN present became reduced by the action of the ethanol and the dehydrogenase.

It will be seen from Table 1 that within the range investigated the equilibrium constant (K_c) is independent of the pH and the concentrations of the reactants. The mean of the twenty-six values found for K_c is $7·19 \times 10^{-9}$ M (range 5·89–8·91). In these measurements the total ionic strengths were very low (approx. 0·04) so that the activity coefficients of all the reactants may be taken as unity. Hence, the thermodynamic equilibrium constant (K_a), which is based on activities rather than concentrations, has in this case the same value as the concentration constant K_c. The ΔG^0 value calculated from the mean value for K_c is 11·11 kcal. and the range of ΔG^0 corresponding to the individual values of K_c is 10·98–11·23 kcal.

The protein concentrations given in Table 1 were obtained from the enzyme activity and Negelein & Wulff's (1937) data for the activity of their purified preparations per mg. protein. This procedure is valid since the purity (activity/optical density at 280 mμ.) of the alcohol dehydrogenase used in the experiments reported here was approximately the same as that of Negelein & Wulff's preparation. Theorell & Bonnischen (1951) have found an apparent effect of high enzyme concentration on the equilibrium of the liver alcohol dehydrogenase system. This may be explained by the assumption that the enzyme combines with reduced in preference to oxidized DPN, so that the total amount of reduced DPN at equilibrium is increased in the presence of high concentrations of enzyme. It will be seen that a 30-fold increase in the enzyme concentration did not appreciably affect the values obtained for K_c in the equilibrium measurements reported in Table 1.

The crystalline yeast alcohol dehydrogenase appears to have been contaminated with small amounts of an enzyme which catalyses the destruction of DPN. At the highest enzyme concentrations 20–40% destruction was observed if the incubation period was prolonged for 30 min. after equilibrium had been attained. It has previously been reported that crystalline enzyme preparations (e.g. ribonuclease, McDonald, 1949) may contain small amounts of other enzymes present as impurities.

Malic dehydrogenase system

$$\text{L-Malate} + \text{DPN}^+ \rightarrow \text{oxaloacetate} + \text{DPNH} + \text{H}^+$$

The equilibrium of this system was measured by adding malic dehydrogenase (1·5–3 μg.) to a solution (3·7 ml.) containing L-malate, DPN and $2·7 \times 10^{-3}$ M-glycine or pyrophosphate buffer. The optical density at 340 mμ. rose rapidly and became almost constant after 3–5 min., the subsequent increase in the next 30 min. being only 3–5%. This slow change was almost certainly due to the spontaneous decomposition of oxaloacetate formed in the reaction. The equilibrium concentration of reduced DPN was obtained from the increase of optical density in the first 10 min. and that of oxaloacetate was assumed to be the same. The concentrations of L-malate and DPN were obtained from the initial concentrations and those of the reduced DPN formed.

Table 1. *Equilibrium in the presence of* iso*propanol, acetone, DPN and alcohol dehydrogenase*

(Temp., 25°; $5{\cdot}5 \times 10^{-3}$ M-pyrophosphate buffer. Equilibrium was approached (*A*) from excess of DPN^+ or, (*B*) from excess of DPNH (see text). Concentrations are final values.)

$$K_c = \frac{[\text{acetone}] \times [\text{DPNH}]}{[\textit{iso}\text{propanol}] \times [\text{DPN}^+]} \times \frac{1}{\text{antilog pH}}.$$

Protein concentration (μg./ml.)	pH	Acetone (M × 10^2)	*iso*Propanol (M × 10^2)	DPNH (M × 10^5)	DPN^+ (M × 10^5)	K_c (M × 10^9)	
27	8·78	19·5	3·92	4·74	5·41	7·23	*A*
27	8·83	19·5	3·92	5·07	5·36	6·95	*B*
27	8·82	29·6	3·90	5·94	8·47	8·05	*A*
27	8·81	29·6	3·90	6·16	8·12	8·91	*B*
54	8·82	19·6	5·87	6·16	3·48	8·90	*B*
54	7·51	3·82	7·80	3·33	7·30	6·88	*A*
54	7·51	3·82	7·80	3·48	7·15	7·36	*B*
54	7·56	1·93	7·80	5·36	4·95	7·45	*A*
54	7·52	1·93	7·80	4·92	4·63	7·95	*A*
54	7·52	1·93	7·80	4·86	4·26	8·55	*B*
38	7·68	1·95	3·93	3·70	5·60	6·85	*A*
38	7·68	1·95	3·93	3·72	5·60	6·86	*B*
22	7·64	1·94	9·78	5·75	3·92	6·66	*A*
22	7·64	1·94	9·78	5·94	3·83	7·05	*B*
43	7·63	1·94	7·81	4·97	4·91	5·90	*A*
43	7·63	1·94	7·81	5·18	4·77	6·34	*B*
43	7·52	3·82	3·92	3·58	15·5	6·80	*A*
43	7·52	3·82	3·92	3·64	15·4	6·95	*B*
43	7·68	3·83	3·93	2·65	7·16	7·52	*A*
43	7·68	3·83	3·93	2·57	7·43	6·99	*B*
36	7·28	1·54	8·49	4·51	6·00	7·16	*A*
72	7·28	1·54	8·49	4·58	6·10	7·16	*A*
290	7·28	1·53	8·41	4·35	5·90	7·02	*A*
290	7·30	1·53	8·41	4·09	5·79	6·45	*A*
720	7·03	1·51	8·33	2·85	6·82	5·89	*A*
720	7·18	1·51	8·33	3·43	5·72	7·20	*A*
					Mean	**7·19**	

Table 2. *Malic dehydrogenase equilibrium*

(Temp., 25°. The first value is the mean of twelve similar measurements which gave values of K_c between $6{\cdot}85 \times 10^{-13}$ and $8{\cdot}75 \times 10^{-13}$ M. Glycine buffer ($2{\cdot}7 \times 10^{-3}$ M) was used in all except the last four measurements where pyrophosphate was used. Concentrations are final values.)

$$K_c = \frac{[\text{DPNH}]\,[\text{oxaloacetate}]}{[\text{DPN}^+]\,[\text{L-malate}]} \times \frac{1}{\text{antilog pH}}.$$

pH	DPNH (=oxaloacetate) (M × 10^5)	DPN^+ (M × 10^5)	L-Malate (M × 10^3)	K_c (M × 10^{13})
8·83	1·45	7·50	5·28	7·87
8·87	1·77	11·8	5·01	7·15
8·95	2·06	11·6	5·28	7·76
8·63	1·77	16·5	5·28	8·41
8·85	2·32	16·0	5·28	9·00
8·87	2·06	16·0	5·13	7·00
8·89	2·42	21·2	5·28	6·75
8·86	2·42	21·2	5·28	7·22
8·81	2·82	32·4	5·27	7·23
8·78	2·86	35·7	5·27	7·22
8·75	2·90	35·8	5·27	7·96
8·83	3·27	41·3	5·14	7·46
8·19	1·53	42·7	5·17	6·86
8·08	1·35	43·1	5·17	6·79
7·55	0·79	43·7	5·19	7·76
			Mean	7·50

No destruction of DPN was detected in this system. As will be seen from Table 2, K_c is practically independent of pH or the concentration of DPN. It was approximately doubled when the concentration of L-malate was increased from about 5×10^{-3} to 5×10^{-2} M. Addition of sodium chloride or sodium fumarate to a reaction system containing 5×10^{-3} M-L-malate also increased K_c. Fumarase was absent so that the fumarate did not cause an apparent increase in K_c by being converted to malate. The increase of K_c accompanying the increased ionic strength can be satisfactorily explained by its effect on the activity coefficients of the reactants. According to the Debye-Hückel limiting law, at very low ionic strengths,

$$\log \gamma = -0{\cdot}5\, z^2 \sqrt{I},$$

where γ is the activity coefficient of an ion of valency z at an ionic strength I. Measurements of Hoskins, Randall & Schmidt (1930), indicate that for the dipolar aspartate^{+2-} and glutamate^{+2-} ions, the limiting law is approximately

$$\log \gamma = -1{\cdot}3 \sqrt{I}.$$

Assuming that the DPN^{+2-} ion also obeys this relation, and applying the limiting law to evaluate the activity coefficients of the other ions in the malic dehydrogenase system, we may obtain for very low ionic strengths,

$$\log K_c = \log K_a + 0{\cdot}7 \sqrt{I},$$

where K_a is the thermodynamic equilibrium constant.

The experimental results are shown in Fig. 2, where $(13 + \log K_c)$ has been plotted against $\sqrt{I}$. Neglecting the two values at the highest ionic strength (0·35), statistical analysis gives a regression line with a slope of 0·770 (S.D. 0·096) and an intercept of 0·777 (S.D. 0·069) at $\sqrt{I} = 0$. The value of the intercept corresponds to $K_a = 6{\cdot}0 \times 10^{-13}$ M and to $\Delta G^0 = 16{\cdot}67$ kcal. (S.D. 0·094 kcal.).

The values obtained at the higher ionic strengths are in good agreement with those of Stern, Ochoa & Lynen (1952) and are within the range of the values given by Schlenk *et al.* (1938).

Reaction with triphosphopyridine nucleotide (TPN). Under the influence of malic dehydrogenase, reduced TPN reacts with oxaloacetate at about 0·03 times the rate of reaction for reduced DPN (Mehler, Kornberg, Grisolia & Ochoa, 1948). An attempt was made to measure the equilibrium constant with TPN for comparison with that obtained using DPN. This was not successful because the rate of reaction between L-malate and TPN was so slow that equilibrium was not attained during the experimental period of 10 hr. The ratio of the initial rates of reaction with DPN and TPN was found to be approximately $6 \times 10^4 : 1$ starting with 0·22 M-L-malate and $1{\cdot}2 \times 10^{-4}$ M-DPN or TPN at pH 9·2; in the reverse direction it was 40:1 (pH 6·9; 8×10^{-5} M-oxaloacetate; 4×10^{-5} M reduced DPN or TPN). The latter ratio agrees with the results of Mehler *et al.* (1948).

Olson & Anfinsen (1953) have found that the equilibrium constant with DPN in the glutamic dehydrogenase system is fairly close to that with TPN. Their results give $K_c = 1{\cdot}45 \times 10^{-13}$ with DPN and $0{\cdot}99 \times 10^{-13}$ with the TPN. The ratio of the two constants are expected to be similar in the malic dehydrogenase system.

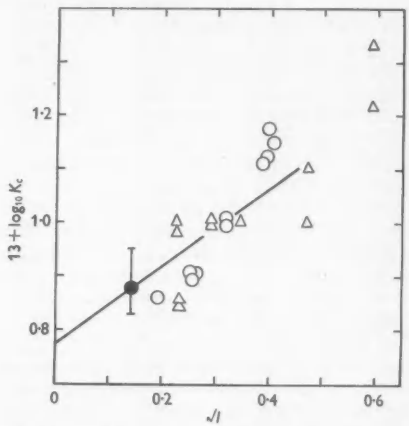

Fig. 2. Effect of ionic strength (I) on the equilibrium constant (K_c) of the malic dehydrogenase system. Temp., 25°; glycine buffer, $2{\cdot}7 \times 10^{-3}$ M; pH, 8·7–8·9; total concentration of DPN, 9×10^{-5} M. ●, range and mean of values shown in Table 1 with approximately 5×10^{-2} M-disodium L-malate: ○, values obtained with higher concentrations of L-malate; △, values with 5×10^{-3} M-L-malate and added NaCl. The equation of the regression line has been calculated omitting the two points at the highest ionic strength.

L-Glycerol 1-phosphate dehydrogenase system

The equilibrium of this system has previously been measured by Euler, Adler & Günther (1937) and by Baranowski (1949). In the following paper (Burton & Krebs, 1953) it will be shown that the equilibrium constant of this system may be used as part of a calculation of ΔG^0 for the hydrolysis of the terminal phosphate groups of adenosinetriphosphate. In view of the importance of a reliable ΔG^0 value for this reaction, and as the data of Euler *et al.* (1937) and of Baranowski are not very consistent, further equilibrium measurements have been made on the glycerol 1-phosphate dehydrogenase system. The product of the dehydrogenation is dihydroxyacetone phosphate (DHAP):

$$\text{L-Glycerol 1-phosphate} + \text{DPN}^+ \rightleftharpoons \text{DHAP} + \text{DPNH} + \text{H}^+. \quad (5)$$

Isomerase and aldolase were also present in the enzyme preparation used so that glyceraldehyde 3-phosphate (GAP) and fructose 1:6-diphosphate (HDP) are also formed:

$$\text{DHAP} \rightleftharpoons \text{GAP}, \quad (6)$$

$$\text{DHAP} + \text{GAP} \rightleftharpoons \text{HDP}. \quad (7)$$

The equilibria of reactions (6) and (7) are such that, at the concentrations attained, dihydroxyacetone phosphate represents about 90 % of the equilibrium mixture.

Equilibrium measurements. The enzyme preparation was added to a solution containing DPN and DL-glycerol 1-phosphate. The final protein concentration was approximately 0·3 μg./ml.; at this concentration the light absorption at 340 mμ. was negligible. In the first 1–2 min. after adding the enzyme, the optical density at 340 mμ. increased rapidly and was then constant at the new level for at least 40 min. No destruction of DPN was detected. The equilibrium concentration of reduced DPN was obtained from the increase of optical density after addition of the enzyme; the concentrations of DPN and L-glycerol 1-phosphate were obtained by difference from those initially present. The concentration of dihydroxyacetone phosphate was obtained from that of reduced DPN by subtracting a correction for the GAP and HDP which were formed by reactions (6) and (7):

$$[\text{DHAP}] = [\text{DPNH}] - [\text{GAP}] - 2\,[\text{HDP}]. \quad (8)$$

These corrections were evaluated using the equilibrium constants of reactions (6) and (7) which have been obtained from the data of Meyerhof & Junowicz-Kocholaty (1943); the value for reaction (7) has been extrapolated to 25° from their data at 30–60°:

$$[\text{DHAP}]/[\text{GAP}] = 22,$$
$$[\text{DHAP}] \times [\text{GAP}]/[\text{HDP}] = 6{\cdot}0 \times 10^{-5}.$$

Even if there are errors of as much as 50 % in each of these values, only a negligible error (4 %) would be introduced into the value obtained for the equilibrium constant of reaction (5). A further correction must be considered for the reaction between triosephosphate and DPN catalysed by the triosephosphate dehydrogenase. However, calculations from the equilibrium data of Meyerhof & Oesper (1947) show that this correction is negligible (<1 %).

The pH was varied between 7·5 and 9·2 using both veronal (diethyl barbituric acid) and pyrophosphate (3×10^{-3} M) buffers. The concentration of L-glycerol 1-phosphate was varied between 10^{-3} and 10^{-2} M, the total concentration of DPN between $1{\cdot}7 \times 10^{-5}$ M and 10^{-4} M and the concentration of reduced DPN between 3 and 5×10^{-5} M.

Sixteen values were obtained for K_c at 25°:

$$K_c = \frac{[\text{DPNH}]\,[\text{dihydroxyacetone phosphate}]}{[\text{DPN}^+]\,[\text{L-glycerol 1-phosphate}]} \times \frac{1}{\text{antilog pH}}.$$

The range of values for K_c is $4{\cdot}81$–$6{\cdot}51 \times 10^{-12}$ M with a mean of $5{\cdot}5 \times 10^{-12}$ M. The ionic strength in most of these experiments was about 0·03. By comparison with the effect of ionic strength on the malic dehydrogenase system (Fig. 2), extrapolation to zero ionic strength gives $K_a = 4{\cdot}6 \times 10^{-12}$ M. These measurements are in good agreement with that of Baranowski (1949) who used a crystalline enzyme preparation free from aldolase, isomerase and triosephosphate dehydrogenase. His data give $K_c = 7 \times 10^{-12}$ M at 22° and at an ionic strength of about 0·15. Euler *et al.* (1937) reported three measurements with a crude enzyme preparation: these give $K_c = 1{\cdot}4$, 5·1 and 5×10^{-12} M respectively, after correction for the glyceraldehyde phosphate and HDP formed and also using the value of Horecker & Kornberg (1948) for the molecular extinction coefficient of reduced DPN.

DISCUSSION

ΔG^0 for the reduction of DPN. This may be obtained from the equilibria in the presence of alcohol dehydrogenase and either (*a*) ethanol and acetaldehyde, or (*b*) *iso*propanol and acetone, together with the free-energy data which are available for these reactants.

As previously reported (Burton, 1952), the thermochemical data for aqueous ethanol and acetaldehyde give $\Delta G^0 = -9{\cdot}68$ kcal. for the reaction

$$\text{CH}_3\text{CHO (aq.)} = \text{CH}_3\text{CH}_2\text{OH (aq.)} + \text{H}_2\,(g). \quad (9)$$

The error in this value may be taken to be less than 0·4 kcal. The measurements of Racker (1950) give $K_c = 1{\cdot}15 \times 10^{-11}$ for the equilibrium with DPN in the presence of crystalline alcohol dehydrogenase at 25° and low ionic strengths (0·04). Assuming the activity coefficients of all the reactants to be unity, $\Delta G^0 = 14{\cdot}94$ kcal. Adding this value to ΔG^0 for reaction (9)

$$\text{DPN}^+ + \text{H}_2 = \text{DPNH} + \text{H}^+;\ \Delta G^0 = 5{\cdot}26 \text{ kcal.} \quad (10)$$

Racker's equilibrium measurements are in good agreement with those made at 20° and higher ionic strengths by Negelein & Wulff (1937) and by Theorell & Bonnischen (1951).

The reliable combustion and entropy data for liquid acetone and *iso*propanol (Miles & Hunt, 1941; Parks, Moseley & Peterson, 1950; Kelley, 1929) give $\Delta G^0 f$ values of $-37{\cdot}18$ and $-43{\cdot}26$ kcal. respectively. By combining these values with those for the vapour pressures (Beare, McVicar & Ferguson, 1930; Butler, Ramchandani & Thomson, 1935) $\Delta G^0 f$ is found to be $-36{\cdot}47$ kcal. for gaseous acetone and $-41{\cdot}57$ kcal. for gaseous *iso*propanol. Hence:

$$\textit{iso}\text{propanol } (g) = \text{acetone } (g) + \text{H}_2\,(g);$$
$$\Delta G^0 = 5{\cdot}10 \text{ kcal.} \quad (11)$$

This is close to the value of 5·035 kcal. which Cubberley & Mueller (1946) have obtained by extrapolation from the data of Kolb & Burwell (1945) for the equilibrium in the presence of a cupric oxide catalyst at 143–218°. It seems unlikely

that the mean of these ΔG^0 values is in error by more than 0·1 kcal. The mean value and partial vapour pressure data (Beare *et al.* 1930; Butler *et al.* 1935) give:

$$\textit{iso}\text{propanol (aq.)} = \text{acetone (aq.)} + H_2\ (g); \quad \Delta G^0 = 5{\cdot}89 \text{ kcal.} \quad (12)$$

From the ΔG^0 values for reactions (4) and (12) the value of $5{\cdot}22 \pm 0{\cdot}2$ kcal. is obtained for reaction (10). This agrees with that obtained from the ethanol-acetaldehyde reaction and corresponds to $E'_0 = -0{\cdot}320 \pm 0{\cdot}004$ V. at pH 7 and 25°. For the same conditions, Borsook (1940) obtained the value of −0·277 by calculation from potentiometric data for the alanine-ammonium pyruvate reaction (Wurmser & Filitti-Wurmser, 1938) and equilibrium data for the alanine-glutamic transaminase (Cohen, 1939) and the glutamic dehydrogenase reactions (v. Euler, Adler, Günther & Das, 1938). These data include at least two major uncertainties. First, the E'_0 value reported for the alanine-ammonium pyruvate reaction (−0·048 V. at pH 7 and 37°) is almost certainly too positive since, as a result of more extensive measurements, Filitti-Wurmser & Morel (1940) give −0·080 V. Secondly, whereas v. Euler *et al.* (1938) consider that under their experimental conditions α-iminoglutarate is the major product of the dehydrogenation of glutamate, recent measurements of Olsen & Anfinsen (1953) indicate that at equilibrium the product is almost wholly ammonium α-ketoglutarate.

ΔG^0 for the reduction of TPN. Olsen & Anfinsen (1953) have measured the equilibria with DPN and also with TPN in the glutamic dehydrogenase system. They found $K_c = 1{\cdot}45 \times 10^{-13}$ with DPN and $0{\cdot}99 \times 10^{-13}$ with TPN. Assuming that the thermodynamic equilibrium constants (K_a) for the two nucleotides are in the same ratio, ΔG^0 for the reduction of TPN may be obtained from the data for DPN, i.e. $\Delta G^0 = 5{\cdot}22 + RT \ln (1{\cdot}45/0{\cdot}99) = 5{\cdot}44$ kcal. Insufficient data are available to make an accurate estimate of the error involved in this assumption, but it probably does not amount to more than $\pm 0{\cdot}4$ kcal. The ΔG^0, $\Delta G'$ and E'_0 values for the dehydrogenation of reduced DPN and TPN, ethanol and *iso*propanol are given in Table 3.

Dehydrogenation of malate, glycerol 1-phosphate, lactate, glyceraldehyde phosphate and isocitrate. ΔG^0 values for these reactions (Table 3) have been calculated from ΔG^0 for the reduction of DPN or TPN and the equilibrium in the presence of the appropriate dehydrogenase. For the malate and glycerol phosphate systems the equilibrium constants are those obtained in this paper extrapolated to zero ionic strength. The equilibrium constant for the glyceraldehyde phosphate system is obtained from the measurements of Meyerhof & Oesper (1947). The value used ($K_c = 8 \times 10^{-9}$) was obtained at low ionic strengths (approx. 0·02; see Figs. 1 and 2 of Meyerhof & Oesper's paper). For the lactate-pyruvate system, Racker's (1950) value for the equilibrium at 25° has been used in preference to that of Kubowitz & Ott (1943) for 22°. If the latter value is corrected to 25° by the data given by Kubowitz & Ott for the effect of temperature on the equilibrium, the value of ΔG^0 is 0·2 kcal. greater than that obtained from Racker's data. The values for the *iso*citrate systems are obtained from Ochoa's (1948) measurements made at a low ionic strength (approx. 0·04).

Most of the ΔG^0 values in Table 3 may be taken to be correct to $\pm 0{\cdot}3$ kcal. The values for the reduction of TPN and the *iso*citrate reactions may be less accurate.

Oxidation-reduction potential measurements. ΔG^0 values for most of the above dehydrogenations have previously been obtained from potential measurements. As will be seen from Table 4, the values obtained from potential measurements are considerably more positive than those evaluated in this paper from equilibrium and thermochemical data.

In the potential measurements, it was necessary to add a reversibly reducible dye to act as a mediator between the enzyme system and the electrode. It is noteworthy that the largest discrepancies occur when indigodisulphonate was the mediator

Table 3. *Free-energy data for some biological dehydrogenations*

(Aqueous solution. Temp., 25°.)

Reductant	Oxidant	ΔG^0 kJ.	ΔG^0 kcal.	At pH 7: $\Delta G'$ (kcal.)	At pH 7: E'_0 (V.)
$DPNH + H^+$	DPN^+	−21·84	−5·22	4·33	−0·320
$TPNH + H^+$	TPN^+	−22·76	−5·44	4·11	−0·324
Ethanol	Acetaldehyde	40·50	9·68	9·68	−0·204
*iso*Propanol	Acetone	24·64	5·89	5·89	−0·296
L-Lactate$^-$	Pyruvate$^-$	43·18	10·32	10·32	−0·190
L-Malate^{2-}	Oxaloacetate^{2-}	47·91	11·45	11·45	−0·166
L-Glycerol 1-phosphate^{2-}	Dihydroxyacetone phosphate^{2-}	42·84	10·24	10·24	−0·192
Glyceraldehyde 3-phosphate$^{2-} + HPO_4^{2-}$	Glyceroylphosphate 3-phosphate^{4-}	24·39	5·83	5·83	−0·286
*dextro-iso*Citrate^{3-}	Oxalosuccinate^{3-}	15·65	3·74	3·74	−0·332
*dextro-iso*Citrate$^{3-} + H^+$	α-Ketoglutarate^{2-}	−53·09	−12·69	−3·14	−0·482

Table 4. *Comparison between ΔG^0 values evaluated in this paper and those obtained from potential measurements*

Reaction (aqueous solution)	Evaluated in this paper ΔG^0 (25°) (kcal.)	Evaluated from potential measurements ΔG^0 (kcal.)	Reference
Ethanol-acetaldehyde	9·68	16·42 (30°)	Lehmann (1934)
*iso*Propanol-acetone	5·89	8·13 (35°)	Wurmser & Filitti (1936)
Lactate-pyruvate	10·32	14·58 (32°)	Baumberger *et al.* (1933)
		11·6 (37°)	Wurmser & Mayer-Reich (1933)
		11·35 (37°)	Banga, Laki & Szent-Györgyi (1933)
		11·44 (35°) 11·76 (25°)	Barron & Hastings (1934)
Malate-oxaloacetate	11·45	12·1 (37°)	Laki (1937)
		15·2 (38°) 15·65 (25°)	Lehmann & Hoff-Jörgensen (1939)
		12 to 15·4 (25°)	Burton*

* These unpublished measurements were made using purified malic dehydrogenase and diaphorase in the presence of nile blue or phenosafranine as mediator. The results, though not reproducible, were all in the range indicated.

(Lehmann, 1934; Baumberger, Jürgensen & Bardwell, 1933; Lehmann & Hoff-Jörgensen, 1939). The measurements of Baumberger *et al.* (1933) have been criticized by Wurmser & Mayer-Reich (1933) and by Barron & Hastings (1934) on the basis that the potential of the indigo disulphonate (−0·125 V. at pH 7 and 30°) is too positive.

The discrepancies between the potentiometric values and those obtained in this paper would be explained if thermodynamic equilibrium had not been attained in the potential measurements. Of the reactions studied in this way, there is only the succinate-fumarate reaction for which the evidence is really convincing that equilibrium was attained (Borsook & Schott, 1931).

SUMMARY

1. The reaction between *iso*propanol and diphosphopyridine nucleotide (DPN) to form acetone and reduced DPN is catalysed by crystalline preparations of yeast alcohol dehydrogenase. About 500 times as much enzyme are required to obtain the same rate of reaction as that with the ethanol-DPN-acetaldehyde system.

2. The equilibrium constant of the isopropanol-DPN-acetone reaction has been combined with reliable free-energy data for *iso*propanol and acetone to calculate the free-energy change for the reduction of DPN. $\Delta G^0 = 5{\cdot}22$ kcal. or 21·84 kJ.. at 25°. This value corresponds to an oxidation-reduction potential E_0' of −0·320 V. at pH 7.

3. The equilibria of the L-malic and L-glycerol 1-phosphate dehydrogenase systems have been measured at low ionic strengths.

4. These and other equilibrium data have been combined with the data for DPN to obtain reliable values (summarized in Table 3) for the free-energy changes for the reduction of triphosphopyridine nucleotide and the dehydrogenations of L-malate, L-lactate, L-glycerol 1-phosphate, glyceraldehyde 3-phosphate and *iso*citrate.

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The Free-energy Changes Associated with the Individual Steps of the Tricarboxylic Acid Cycle, Glycolysis and Alcoholic Fermentation and with the Hydrolysis of the Pyrophosphate Groups of Adenosinetriphosphate

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Now that the main stages of the energy-producing reactions in living matter are known, investigations into biological energy transformations are likely to become increasingly concerned with the mechanisms by which the free energy of respiration and fermentation is converted into work. In many of such studies it is essential to possess accurate data on the free-energy changes associated with the individual steps of the energy-giving reactions and the hydrolysis of energy-rich phosphate compounds. Reliable data for many of these reactions are still lacking. Many free-energy data which have hitherto been used, e.g. by Lipmann (1941, 1946), Kalckar (1941), Ogston & Smithies (1948), Dickens (1951), Ochoa (1951), Kaplan (1951), Ogston (1951) and Oesper (1951) are not more than approximate assessments.

The preceding papers report new equilibrium measurements for the fumarase, aconitase and transaminase enzyme systems (Krebs, 1953*a*, *b*) and certain nicotinamide nucleotide dehydrogenase systems (Burton & Wilson, 1953). In this paper, the free-energy changes calculated from these and other equilibrium measurements are used in conjunction with free-energy data derived from literature data on heats of combustion and heat capacities. The newer data make it possible to evaluate more accurately than before the free-energy changes associated with each step of the biological degradation of carbohydrate.

Section 1 is concerned with the critical selection of thermochemical and ancillary data for pure substances of biological interest in order to calculate the

best available values for the free energies of formation. In section 2 the free-energy changes are evaluated for the steps of the tricarboxylic acid cycle, in section 3 for the hydrolysis of the terminal phosphate groups of adenosinetriphosphate (ATP) and in section 4 for the glycolytic reactions.

THERMODYNAMIC SYMBOLS AND CONVENTIONS

The symbols used in this paper are based on the conventions of Lewis & Randall (1923) and the Report of the Royal Society Committee on Symbols (1951). They are as follows:

ΔG	Increment of free energy for the given reaction under specified conditions (ΔF of Lewis & Randall, 1923).
ΔG^0	ΔG for standard conditions, i.e. the reactants in their standard states.
$\Delta G'$	ΔG for a reaction in solution with all reactants except the hydrogen ion in their standard states.
$\Delta G^0 f$	Free energy of formation of a compound from its elements under standard conditions.
$\Delta H^0 f$	Heat of formation of a compound under standard conditions.
$\Delta S^0 f$	Entropy of formation of a compound under standard conditions.
K_a	Thermodynamic equilibrium constant, i.e. the product of the activities of the reactants on the right-hand side divided by the corresponding product for the left-hand side when the reaction is at equilibrium.
K_c	Equilibrium constant based on concentrations instead of activities.
I	Ionic strength.
m	Molality (moles/1000 g. water).
γ	Molal activity coefficient (i.e. activity/molality).
$\boldsymbol{R}$	Gas constant (8·3144 abs. joules/deg./mole or 1·9872 cal./deg./mole).
T	Absolute temperature.

Some of the more important values in the tables are given both in kilocalories (kcal.) and in kilojoules (kJ.), but in the text the kilocalorie is used throughout. By definition 1 thermodynamical calorie is equal to 4·1840 absolute joules. The standard states (unit activity) are a defined physical form for pure substances (e.g. liquid water) and the ideal molal solution for dissolved reactants. The relation between ΔG^0 and K_a is

$$\Delta G^0 = -\boldsymbol{R}T \ln K_a.$$

At 25°, $\Delta G^0 = -(1{\cdot}3643 \log K_a)$ kcal. or $-(5{\cdot}708 \log K_a)$ kJ. $\Delta G = \Delta G^0 + \boldsymbol{R}T$ ln product of activities of reactants on right-hand side/product of activities of reactants on left-hand side. Unless otherwise stated, the data refer to 25°. All free-energy changes are expressed per mole. In accordance with the usual custom, most of the free-energy values in this paper are given to 0·01 kcal. Although some of the equilibrium data justify this order of accuracy, most of the values which are based on thermal measurements are not certain to the first decimal place (0·1 kcal.). However, two decimal places are retained for the purposes of calculation.

SECTION 1

Thermochemical free-energy data

The standard free energies of formation for several substances of physiological importance are given in Table 1. These have been obtained from what appear to be the most reliable thermochemical and ancillary data which are available. In most cases the free energy of formation ($\Delta G^0 f$) for the pure compound is obtained by the relation

$$\Delta G^0 f = \Delta H^0 f - T\Delta S^0 f,$$

where $\Delta H^0 f$ and $\Delta S^0 f$ respectively are the heats and entropies of formation, and T is the absolute temperature. $\Delta H^0 f$ and $\Delta S^0 f$ have been calculated from data for the heat of combustion and the entropy using values for $\Delta H^0 f$ of CO_2 and H_2O and the entropies of the elements in the standard states as given by Rossini, Wagman, Evans, Levine & Jaffe (1952). Where no value is given for $\Delta S^0 f$, $\Delta G^0 f$ has been evaluated from $\Delta H^0 f$ and spectroscopic data. The value for $\Delta H^0 f$ of carbon dioxide differs from that used by earlier authors (e.g. Parks & Huffman, 1932; Borsook & Huffman, 1938) by 0·19 kcal.; this accounts for some of the differences between the $\Delta G^0 f$ values obtained here and those previously given for the same compounds.

Reliable thermochemical data are also available for other amino-acids and peptides (Borsook & Huffman, 1938; Huffman, 1942), several purines (Stiehler & Huffman, 1935) and a number of sugars (Clarke & Stegeman, 1939; Anderson & Stegeman, 1941; Jack & Stegeman, 1941).

Sources of the data. Those for acetaldehyde, acetone and ethanol are as previously described (Burton, 1952).

There is considerable uncertainty (± 1 kcal.) in the available data for the heat of combustion of acetic acid; the value used is that selected by Bichowsky & Rossini (1936). The entropy value of acetic acid is that of Parks & Huffman (1932).

ΔG^0 for the solution of acetic acid in water was calculated from the vapour pressure measurements of Fredenhagen & Liebster (1932) and data given by Halford (1941) for the equilibrium between dimeric and monomeric acetic acid in the vapour phase.

Table 1. *Thermochemical free-energy data*

(All values are per mole of substances at 25°. Unless otherwise stated, data for pure compounds refer to the crystalline material.)

Compound	$-\Delta H^0f$ (kcal.)	$-\Delta S^0f$ (cal./deg.)	$-\Delta G^0f$ (kcal.)	ΔG^0 of solution in water (kcal)	ΔG^0 of ionization (kcal.)	$-\Delta G^0f$ in aqueous solution (kcal.)	$-\Delta G^0f$ in aqueous solution (kJ.)
Acetaldehyde (gas)	39·76	—	31·96	−1·75	—	33·71	141·04
Acetic acid (liq.)	116·44	76·1	93·75	−1·73	—	95·48	399·49
Acetate	—	—	—	—	6·49	88·99	372·33
Acetone (liq.)	59·34	74·32	37·18	−1·34	—	38·52	161·17
DL-Alanine	134·72	153·7	88·89	−0·35	—	89·24	373·38
L-Alanine	134·17	153·7	88·34	−0·35	—	88·69	371·08
						88·83*	371·66*
						88·75†	371·33†
NH_3 (gas)	—	—	3·98	−2·39	—	6·37	26·65
NH_4^+	—	—	—	—	−12·63	19·00	79·50
L-Aspartic acid	233·75	194·1	174·88	2·45	—	172·43	721·45
L-Aspartate^{+2-}	—	—	—	—	5·32	167·11	699·19
n-Butanol	79·59	131·5	40·39	−0·68	—	41·07	171·84
Butyric Acid (liq.)	128·09	125·2	90·65	−0·55	—	91·2	381·58
Butyrate	—	—	—	—	6·58	84·6	353·97
CO_2 (gas)	94·052	—	94·26	2·00	—	92·26	386·02
HCO_3^-	—	—	—	—	8·66	140·29	586·97
Ethanol (liq.)	66·34	82·4	41·77	−1·62	—	43·39	181·54
Fumaric acid	194·13	126·2	156·49	1·82	—	154·67	647·14
Fumarate^{2-}	—	—	—	—	10·26	144·41	604·21
α-D-Glucose	304·64	291·7	217·56	−1·82	—	219·38	917·89
L-Glutamic acid	240·38	222·4	174·05	2·05	—	172·00	719·65
L-Glutamate^{+2-}	—	—	—	—	5·89	166·11	695·00
Glycerol (liq.)	159·80	153·6	114·02	−2·74	—	116·76	488·52
Methane (gas)	17·89	—	12·14 (50·79 kJ.)	—	—	—	—
Succinic acid	224·92	155·1	178·68	0·29	—	178·39	746·38
Succinate^{2-}	—	—	—	—	13·42	164·97	690·23
Water (liq.)	68·317	—	56·69 (237·19 kJ.)	—	—	—	—

* Calculated from value for DL-alanine. † Value adopted in this paper.

The combustion and entropy data for alanine, aspartic acid and glutamic acid and also ΔG^0 for the solution of L- and DL-alanine are those given by Borsook & Huffman (1938). It is noteworthy that the ΔG^0f of aqueous L-alanine is within 0·14 kcal. of that obtained from the ΔG^0f of aqueous DL-alanine and ΔG^0 for the racemization ($-RT\ln 2 = 0{\cdot}41$ kcal.). The mean (−88·75 kcal.) of the two values for aqueous L-alanine is adopted for subsequent calculations.

The ΔG^0 values for the solution and ionization of aspartic and glutamic acid differ from those given by Borsook & Huffman (1938); they are obtained as follows: at 25° a saturated solution of L-aspartic acid contains 0·0375 mole/1000 g. water (see Borsook & Huffman, 1938) and from the relevant ionization constant ($1{\cdot}24 \times 10^{-4}$), 95% or 0·0355 molal remains undissociated. Hoskins, Randall & Schmidt (1930) have measured the activity coefficient (γ) of aspartic acid in aqueous solution at 0° and at concentrations up to $m = 0{\cdot}03$. The curve obtained by plotting $\log \gamma$ against m has been extrapolated to $m = 0{\cdot}0355$. Hence γ for the undissociated acid is found to be 0·45. By applying the relation $\mathrm{d}\ln\gamma/\mathrm{d}T = -(\bar{H} - \bar{H}^0)/RT^2$, where $(\bar{H} - \bar{H}^0)$ is the relative partial molal heat content (190 cal./mole; Borsook & Huffman, 1938) it will be seen that the temperature correction to 25° is negligible. ΔG^0 of solution is therefore $-RT\ln\gamma m = -RT\ln(0{\cdot}45 \times 0{\cdot}0355) = 2{\cdot}45$ kcal. The value obtained by Borsook & Huffman is 2·55 kcal. To obtain ΔG^0 of ionization, the thermodynamic ionization constant has been taken to be $1{\cdot}24 \times 10^{-4}$ corresponding to pK = 3·90 (Schmidt, 1938; Batchelder & Schmidt, 1940; Smith & Smith, 1942).

The procedure for L-glutamic acid is similar. The saturated solution has been taken to be 0·0595 molal at 25° and the acid to be 96·2% undissociated. The activity data of Hoskins *et al.* (1930) have been extrapolated to $0{\cdot}057m$ undissociated acid, where γ is found to be 0·55. Hence ΔG^0 of solution is $-RT\ln(0{\cdot}55 \times 0{\cdot}057) = 2{\cdot}05$ kcal. The value obtained by Borsook & Huffman is 2·3 kcal. To calculate ΔG^0 of ionization the appropriate pK has been taken as 4·315 (Neuberger, 1936).

The data for NH_3 and NH_4^+ are from Rossini *et al.* (1952). For *n*-butanol the heat of combustion is Rossini's (1934) value and the entropy value used is that given by Parks &

Huffman (1932). ΔG^0 of solution is obtained from data of Butler, Ramchandani & Thomson (1935). The combustion and entropy data for butyric acid are those selected by Parks & Huffman (1932); ΔG^0 of solution is an estimated value and ΔG^0 of ionization is obtained from data of Saxton & Darken (1940).

The ΔG^0f and ΔH^0f values for carbon dioxide, water and methane are from Rossini *et al.* (1952). ΔG^0 for the solution of CO_2 is obtained from the solubility data of Harned & Davis (1943). ΔG^0f for the HCO_3^- ion is calculated from the values for water and aqueous carbon dioxide and the ionization constant as measured by Harned & Davis.

The combustion and entropy data used for succinic and fumaric acids are those given by Huffman & Fox (1938). The ΔG^0 values for solution and ionization are those of Borsook & Schott (1931); in the case of succinic acid more recent measurements of the ionization constants (Pinching & Bates, 1950) and of the activity coefficients in aqueous solution (Robinson, Smith & Smith, 1942) confirm the data used by Borsook & Schott.

For glucose the combustion and entropy data for anhydrous α-D-glucose given by Huffman & Fox (1938) are used. When glucose crystallizes from aqueous solution at 25°, the solid form obtained is the monohydrate and not the anhydrous form. Huffman & Fox (1938) extrapolated the solubility data for the anhydrous form at 50–100° (Jackson & Silsbee, 1922) and obtained the value of 0·162 for the mole ratio of glucose (moles glucose/mole water) in an aqueous solution which is in equilibrium with the anhydrous form at 25°. In order to obtain ΔG^0 of solution it is necessary to know the activity coefficient (γ) of glucose in this solution. An approximate value for γ may be obtained by extrapolating data for lower concentrations which may be calculated (Table 2) from the osmotic pressure measurements of Berkeley & Hartley (1906). A straight line is obtained by plotting $1/\gamma$ against the mole ratio. By extrapolation, $\gamma = 2{\cdot}4$ at a mole ratio of 0·162. Hence for the solution of anhydrous glucose at 25°,

$$\Delta G^0 = -RT \ln (2{\cdot}4 \times 0{\cdot}162/0{\cdot}0180) = -1{\cdot}82 \text{ kcal.}$$

(0·0180 is the mole ratio of a molal solution).

Table 2. *Activity of glucose in aqueous solution at* 0°

(Calculated from osmotic pressure data of Berkeley & Hartley (1906). Concentrations per 100 ml. have been converted to mole ratios with the aid of density data of Jackson (1917). For water, unit activity is the pure liquid; for glucose, it is the ideal molal solution. The activity of the glucose was calculated from that of the water by the method of Lewis & Randall (1923).)

Glucose concn. (g./100 ml.)	Osmotic pressure (atm.)	Mole ratio of glucose	Activity of water	Activity coefficient of glucose
9·98	13·21	0·0106	0·989	1·02
19·95	29·17	0·0226	0·976	1·11
31·92	53·19	0·0401	0·958	1·16
44·82	87·87	0·063	0·930	1·30
54·86	121·18	0·085	0·907	1·38

The data for glycerol are derived from the heat of combustion of Parks, West, Naylor, Fujii & McClaine (1946) and the entropy value given by Ahlberg, Blanchard & Lundberg (1937). ΔG^0 of solution is obtained from vapour pressure data for aqueous solutions of glycerol. Since the vapour pressure of 98·43 % glycerol is 0·536 mm. Hg at 13° (Daudt, 1923) while that of anhydrous glycerol is 0·0025 mm. Hg at 50° (Stedman, 1928), less than 0·5 % of the vapour pressure of the 98·43 % solution is due to the glycerol and the remainder is due to the water component. Hence, if p is the vapour pressure of the solution, and p_0 that of pure water at the same temperature, the relative vapour pressure (p/p_0) may be taken as the thermodynamic activity of the water present in the glycerol solution. This has been evaluated for a range of glycerol concentrations using vapour pressure data of Scatchard, Hamer & Wood (1938) for solutions up to 56·5 % glycerol, those of Fricke (1929) for higher concentrations (up to 98 %) and the value of Daudt for 98·43 % glycerol. When p/p_0 is plotted against the glycerol concentration, these measurements are very consistent, not only with each other, but also with those of Grover & Nicol (1940) at 25° and the values of Dieterici (at 0°) as compiled in the *International Critical Tables* (1931). They do not agree with the values of Drucker & Moles (1909) nor with the values given by Dulitskaya (1945) for 25°. In contrast to the other data neither of these two series of results falls on a smooth curve. The activity of glycerol in aqueous solution is obtained from the activity of the solvent by integration according to the procedure of Lewis & Randall (1923). At 15°, the value obtained for the activity of anhydrous glycerol is 111 times that of an ideal molal solution in water. ΔG^0 of solution is therefore $-RT \ln 111 = -2{\cdot}695$ kcal. The value at 25° (2·74 kcal.) is obtained from this and the heat of solution ($-1{\cdot}38$ kcal./mole; Fricke, 1929). Any uncertainty in this value is mainly due to errors in the data for the higher concentrations (>90 %) of glycerol. Even if the error at these concentrations amounted to 50 % of the vapour pressure the error in ΔG^0 would be only 0·07 kcal. The values used for the activity of both water and glycerol in solutions more concentrated than 0·25 mole glycerol/mole water are given in Table 3. Data for lower concentrations are given by Scatchard *et al.* (1938).

Table 3. *Activity of glycerol in aqueous solution*

(Based mainly on vapour pressure data of Fricke (1929) for 15°. p/p_0 = vapour pressure of solution relative to that of pure water. For water, unit activity is pure water; for glycerol, it is the ideal molal solution.)

Mole ratio of glycerol	p/p_0 = Activity of water	Activity of glycerol
0·250	0·763	14·9
0·30	0·724	18·4
0·40	0·650	25·0
0·50	0·595	30·6
0·70	0·498	41·1
1·00	0·422	50·3
1·50	0·312	64
2·00	0·264	71
5·0	0·124	91
10·0	0·062	101
∞	—	111

Accuracy of the ΔG^0f values. It is difficult to give precise limits of the errors involved, but it is useful to consider the following estimates when the data are used in subsequent calculations.

The reliability of the ΔG^0f values depends primarily on the accuracy with which the heat of

combustion has been measured. In the case of glucose an error of 0·03 % in the heat of combustion would introduce an error of 0·2 kcal. into ΔH^0f and hence into ΔG^0f. An error of 0·5 % in the entropy would also introduce an error of 0·07 kcal. into ΔG^0f. Except for the heat of combustion of acetic acid and possibly that of butyric acid, the combustion and entropy data are of at least this order of accuracy. The simpler compounds (ammonia, carbon dioxide, water and methane) have been very carefully studied, and in each case the error is almost certainly less than 0·05 kcal. The uncertainties in the ΔG^0f values for aqueous glucose, aspartic and glutamic acids and the aspartate and glutamate ions are probably about $\pm$ 0·4 kcal. The values given for the other compounds, with the exception of acetic and butyric acids, are likely to be correct to $\pm$ 0·2 kcal.

Inconsistencies in the free-energy data

Some apparent inconsistencies have come to light during this work and require further investigation. They are as follows:

Formate. ΔG^0f as evaluated from Woods's (1936) measurements of the formic hydrogenlyase equilibrium is $-83·77$ kcal. whereas $-80·0$ kcal. is obtained from thermochemical and ancillary data (Rossini *et al.* 1952).

Sucrose. Data for the heat of combustion (Clarke & Stegeman, 1939) and entropy (Parks, Huffman & Barmore, 1933), together with those for ΔG^0 of solution (Scatchard *et al.* 1938), give the value of $-370·90$ kcal. for ΔG^0f of aqueous sucrose.

This value combined with that for aqueous glucose and ΔG^0 for the conversion of glucose to fructose (0·44 kcal. see section 4) gives $\Delta G^0 = -10·8$ kcal. for the inversion of sucrose. However, Doudoroff's (1943) equilibrium data for the sucrose phosphorylase reaction, together with the value of ΔG^0 obtained for the hydrolysis of glucose 1-phosphate (see section 4), indicate that ΔG^0 of inversion is $-6·5$ kcal. It is unlikely that the discrepancy can be accounted for by errors in the combustion measurements. The values used for the heats of combustion of sucrose and of glucose, together with the value of Clarke & Stegeman (1939) for fructose and data for the heats of solution of anhydrous glucose (Hendricks, Dorsey, LeRoy & Moseley, 1930), fructose (Hendricks, Steinbach, LeRoy & Moseley, 1934) and sucrose (Higbie & Stegeman, 1950), indicate that ΔH of inversion is $-4·9$ kcal.; this is in fair agreement with the measured value of $-3·56$ kcal. (Sturtevant, 1937).

Lactose. Two independent but inconsistent values for ΔG^0f of aqueous lactose may be obtained starting from the combustion and entropy data for α-lactose monohydrate and anhydrous β-lactose. The data of Clarke & Stegeman (1939) and of Anderson & Stegeman (1941) give $-419·11$ kcal. for ΔG^0f of the solid α-lactose monohydrate and $-374·56$ kcal. for solid anhydrous β-lactose. The solubility data for the two forms (Hudson, 1908) indicate that ΔG^0 of solution to form the α–β equilibrium mixture is 0·27 kcal. for the monohydrate and about $-0·7$ kcal. for the anhydrous form. The data for the monohydrate give $(-419·11 + 0·27 - \Delta G^0f$ of liquid water$) = -362·15$ kcal. for the ΔG^0f value of aqueous lactose, while the data for the anhydrous form give $-374·56 - 0·7 = -375·26$ kcal.

Maltose. The thermochemical measurements of Anderson & Stegeman (1941) and Clarke & Stegeman (1939) for β-maltose monohydrate, together with the solubility data of Dehn (1917), give the ΔG^0f value of $-357·80$ kcal. for aqueous maltose. This indicates that ΔG^0 for the hydrolysis of aqueous maltose is $-23·47$ kcal. However, the equilibrium data of Monod & Torriani (1950) for the amylomaltose system suggest that ΔG^0 for the hydrolysis of maltose is approximately the same as for the hydrolysis of a glucosidic link of starch. In section 4 of this paper this is estimated to be $-4·2$ kcal.

SECTION 2

Free energies of formation for intermediates of the tricarboxylic acid cycle and associated metabolites

As stated in section 1, ΔG^0f values based on thermochemical measurements are available for two intermediates of the cycle, namely, succinate and fumarate, and also for three metabolites related to the cycle, namely, alanine, aspartate and glutamate (see Table 1). ΔG^0f values for other intermediates of the cycle, obtained from the values for the above five substances, and equilibrium data are given in Table 4. Details of the procedure are as follows.

Activity coefficients. It is necessary in the calculations to consider the activity coefficients of the reactants in the equilibrium mixtures. For most non-electrolytes the values of these coefficients may be taken as very close to unity even at concentrations as high as 1 M. If, as in many cases, the reactions involve electrolytes and the measurements were made at an ionic strength $I > 0·1$, the coefficients are expected to be markedly different from unity. In many of the reactions considered, ions of similar size, valency and charge distribution appear on opposite sides, e.g. the fumarase reaction

$$\text{fumarate}^{2-} + H_2O = \text{malate}^{2-}. \qquad (1)$$

The activity coefficients may be assumed to be identical for similar ions in the same solution and the coefficients may in these cases be taken to cancel each other in the expression for the thermochemical equilibrium constant (K_a).

If the ionic strength is less than 0·05 M, fairly reliable values can be assessed for the activity coefficients of most electrolytes, since at low ionic strengths the activity coefficients are almost identical for salts of the same valency type. If measurements of enzyme-catalysed equilibria are therefore carried out at low ionic strengths, the uncertainties due to activity coefficients are reduced to a minimum. However, some equilibrium measurements have been carried out in the presence of a buffer which may contribute as much as 0·1 to the ionic strength of the equilibrium mixture.

Fumarate. The accuracy of most of the free-energy data calculated in this section depends on the accuracy of the ΔG^0f value for fumarate which serves as a reference substance. The value given in Table 1 is obtained from modern thermochemical data, and its reliability is confirmed by the fact that it is reasonably consistent with free-energy data for both the succinic dehydrogenase and the aspartase reactions. For example, the potential measurements of Borsook & Schott (1931) for the reaction

$$\text{Succinate} \rightarrow \text{fumarate} + H_2$$

indicate that ΔG^0 is 20·16 kcal., whereas the ΔG^0f values for succinate and fumarate (Table 1) give 20·56 kcal. The discrepancy of 0·40 kcal. is within the limits of error of the thermochemical and ancillary data but is somewhat larger

Table 4. *Values for the free energy of formation (ΔG^0f) for metabolites associated with the tricarboxylic acid cycle*

(The data refer to aqueous solution and 25° and are derived from equilibrium measurements and ΔG^0f values for other metabolites given in Table 1. The values for acetoacetate and β-hydroxybutyrate are less reliable than the other values.)

	$-\Delta G^0f$	
	kcal.	kJ.
Acetoacetate$^-$	115	481·16
cis-Aconitate^{3-}	215·17	900·27
Citrate^{3-}	273·90	1145·10
*iso*Citrate^{3-}	272·31	1139·34
β-Hydroxybutyrate$^-$	121	506·26
α-Ketoglutarate^{2-}	190·74	798·06
Lactate$^-$	123·64	517·31
Malate^{2-}	201·98	845·08
Oxaloacetate^{2-}	190·53	797·18
Oxalosuccinate^{2-}	268·57	1123·70
Pyruvate$^-$	113·32	474·13

than the uncertainty of the potentiometric ΔG^0 value (possibly 0·07 kcal.). Since it is uncertain what fraction of the error lies in the value for succinate and in that for fumarate, no correction is attempted. For the aspartase reaction

$$\text{aspartate} \rightarrow \text{fumarate} + NH_4^+$$

the ΔG^0f values of the reactants give $\Delta G^0 = 3{\cdot}70$ kcal. (25°), whereas Borsook & Huffman (1938) obtained 3·72 kcal. (37°) using the equilibrium data of Woolf (1929).

Malate, oxaloacetate, pyruvate and α-ketoglutarate. Starting from the value for fumarate ΔG^0f values may be obtained for malate and oxaloacetate. For the fumarase reaction (1) Krebs (1953*a*) found $K_c = 4{\cdot}42$ at 25°. Assuming that the activity coefficients of fumarate and malate cancel out, $\Delta G^0 = -RT \ln K_c = -0{\cdot}88$ kcal. Hence, for malate^{2-}, $\Delta G^0f = -144{\cdot}41 - 56{\cdot}69 - 0{\cdot}88 = -201{\cdot}98$ kcal. The value for oxaloacetate is obtained from the reaction

$$\text{malate}^{2-} = \text{oxaloacetate}^{2-} + H_2,$$

$\Delta G^0 = 11{\cdot}45$ kcal. (Burton & Wilson, 1953).

Data for the pyruvate$^-$ and α-ketoglutarate^{2-} ions may be obtained (cf. Darling, 1945, 1947) with the help of equilibrium data for the transaminase systems

L-glutamate + oxaloacetate = L-aspartate + α-ketoglutarate, (2)

L-glutamate + pyruvate = L-alanine + α-ketoglutarate. (3)

According to Krebs (1953*b*), at 25°, $K_c = 6{\cdot}74$ for reaction (2) and 1·52 for reaction (3). These values are converted to thermodynamic equilibrium constants as follows. The measurements for reaction (2) were carried out at an ionic strength (I) of 0·152. The activity coefficients (γ) of the aspartate^{+2-} and glutamate^{+2-} ions in the equilibrium mixture are assumed to be the same as in solutions of the respective monosodium salts at the same ionic strength. These have been measured by Hoskins *et al.* (1930) for concentrations up to $m = 0{\cdot}06$. The curve obtained by plotting $\log \gamma$ against $\sqrt{m}$ has been extrapolated to $m = I = 0{\cdot}152$. The values of γ thus obtained are 0·655 for sodium aspartate and 0·615 for sodium glutamate respectively. Hence

$$\frac{\gamma_{\text{aspartate}^{+2-}}}{\gamma_{\text{glutamate}^{+2-}}} = \frac{(\gamma_{\text{sodium aspartate}})^2}{(\gamma_{\text{sodium glutamate}})^2} = 1{\cdot}15.$$

For the α-ketoglutarate ion, γ is assumed to be the same as for the oxaloacetate ion. Hence,

$$\Delta G^0 = -RT \ln (1{\cdot}15 \times K_c) = -1{\cdot}21 \text{ kcal.}$$

ΔG^0f for α-ketoglutarate is obtained from this value and the ΔG^0f values for glutamate, aspartate and oxaloacetate.

The equilibrium measurements for the glutamate-pyruvate transamination (3) were carried out at $I = 0{\cdot}163$ with approximately 0·0123 M-L-alanine. According to Smith & Smith (1937), γ for aqueous L-alanine is 1·00 at this concentration, but Joseph (1939) has shown that it is slightly decreased by the presence of salts; γ is therefore taken to be 0·95. For sodium pyruvate and sodium α-ketoglutarate γ is assumed to be the same as for sodium acetate and sodium fumarate, respectively, at the same total ionic strength. The respective values for the mean activity coefficients of these salts ($I = 0{\cdot}163$) are 0·755 (Smith & Robinson, 1942) and 0·545 (Robinson *et al.* 1942). For sodium glutamate, the above extrapolation of the results of Hoskins *et al.* (1930) indicates that γ is equal to 0·61. The quotient of the activity coefficients is therefore

$$\frac{\gamma_{\text{alanine}^{+-}} \times \gamma_{\alpha\text{-ketoglutarate}^{2-}}}{\gamma_{\text{glutamate}^{+2-}} \times \gamma_{\text{pyruvate}^-}} = \frac{\gamma_{\text{alanine}^{+-}} \times (\gamma_{\text{disodium }\alpha\text{-ketoglutarate}})^3}{(\gamma_{\text{sodium glutamate}})^2 \times (\gamma_{\text{sodium pyruvate}})^2} = \frac{0{\cdot}95 \times (0{\cdot}545)^3}{(0{\cdot}61)^2 \times (0{\cdot}755)^2} = 0{\cdot}73.$$

Hence, for reaction (3)

$$\Delta G^0 = -RT \ln (0{\cdot}73 \times K_c) = -0{\cdot}06 \text{ kcal.}$$

The data for reactions (2) and (3) indicate that

$$\Delta G^0 = -1{\cdot}15 \text{ kcal.}$$

for the reaction

L-alanine + oxaloacetate = L-aspartate + pyruvate. (4)

A value is thus obtained for ΔG^0f of the pyruvate ion ($-113{\cdot}32$ kcal.) using the values for alanine, oxaloacetate and aspartate. It is reasonably consistent with Blaschko's (1925) measurement of the heat of combustion of pyruvic acid and an assumed entropy of 54·7 (cf. Lipmann, 1941).

The values used for the activity coefficients of the glutamate and aspartate ions in the above calculation may have to be modified when more comprehensive activity data are available for sodium glutamate and aspartate, especially in the presence of other electrolytes. Nevertheless, it is unlikely that uncertainties in the activity coefficients introduce errors into the ΔG^0 values obtained for reactions (2) and (3)

of more than 0·05 and 0·15 kcal. respectively. These errors are not expected to be additive when reaction (4) is considered; the error from all causes in ΔG^0 for this reaction is probably less than 0·2 kcal.

Tricarboxylic acids. Starting from ΔG^0f for α-ketoglutarate, values may be obtained for *iso*citrate and oxalosuccinate using the free-energy changes for the dehydrogenation of *iso*citrate and its subsequent decarboxylation to α-ketoglutarate (cf. Kaplan, 1951). The ΔG^0 values used for these reactions are those calculated by Burton & Wilson (1953) from their value for ΔG^0 of reduction of triphosphopyridine nucleotide (TPN) and Ochoa's (1948) equilibrium measurements for the *iso*citric dehydrogenase and oxalosuccinic carboxylase systems. Since these measurements were performed at low ionic strengths (approx. 0·04) no correction is necessary for the activity coefficients. The instability of oxalosuccinate precluded accurate measurement of the equilibrium concentrations; hence the ΔG^0f value for this substance is less accurate than that for *iso*citrate.

ΔG^0f values for citrate and *cis*-aconitate are obtained from the *iso*citrate value using Krebs's (1953*a*) equilibrium data for the aconitase system. In evaluating ΔG^0 for the aconitase reactions, the activity coefficients of the trivalent anions have been assumed to be the same for the three tricarboxylic acids.

Lactate. ΔG^0f for the lactate ion (−123·64 kcal.) is obtained from the value for pyruvate and ΔG^0 for the dehydrogenation of lactate (Burton & Wilson, 1953). The available heat of combustion and entropy data (Parks & Huffman, 1932) give a ΔG^0f value of −123·74 kcal. for liquid DL-lactic acid. This corresponds to about −121 kcal. for the aqueous L-lactate ion. The discrepancy of 2·5 kcal. between the two values for the lactate ion, is too large to be entirely explained by inaccuracies in the equilibrium constants. It is almost certainly due to an inaccuracy in the value used for the heat of combustion which is based on measurements of Emery & Benedict (1911) and of Meyerhof (1922). Many of the heats of combustion values obtained in this period have had to be revised by as much as 2 kcal./mole (cf. Rossini, 1932). The value of −123·64 kcal. obtained from equilibrium measurements is therefore adopted for the ΔG^0f of the lactate ion.

Acetoacetate and β-hydroxybutyrate. Approximate ΔG^0f values for these compounds may be obtained starting from the value for butyric acid (Table 1). Inspection of the ΔG^0f values for several compounds especially glycerol, *n*- and *iso*-propanol, malic acid and succinic acid suggest that the introduction of an oxygen atom into aqueous butyric acid to form β-hydroxybutyric acid involves a decrease of 36±1 kcal. in the ΔG^0f. The value for aqueous β-hydroxybutyric acid is therefore about −127·5 kcal. and that for the anion about −121 kcal. Combining this value for β-hydroxybutyric with the potential measurements of Hoff-Jörgensen (1938) for the β-hydroxybutyric dehydrogenase reaction, ΔG^0f is found to be −115 kcal. for the acetoacetate ion. A similar value may be obtained on the assumption that the free-energy change of the reaction

$$\text{acetone} + CO_2 \rightarrow \text{acetoacetate}^- + H^+$$

is similar to those for the analogous carboxylations of pyruvate to oxaloacetate and of α-ketoglutarate to oxalosuccinate for which the $\Delta G'$ values are 7·50 and 6·88 kcal. respectively at pH 7. Taking the value of 7 kcal. for the carboxylation of acetone, ΔG^0f is −116 kcal. for acetoacetate.

The individual stages of the tricarboxylic acid cycle and associated reactions

Free-energy changes for the individual steps of the cycle and certain associated reactions are given in Table 5. With the exception of the values for the reactions involving the coenzyme of acetylation (CoA) they are derived from the data presented in the preceding sections of this paper. Since the conventional thermodynamic standard states are very different from the physiological conditions the free-energy changes are also given for the following concentrations of reactants: pH 7, 0·01 M concentration of all dissolved reactants except the H^+ ion, 0·2 atm. oxygen and 0·05 atm. carbon dioxide. The free-energy changes computed for the concentrations of intermediates found by Frohman, Orten & Smith (1951) in rat tissues are very similar to those given in Table 5. Since not all of the free-energy data are accurate enough to justify the small corrections thus introduced it is preferable at this stage to give them for the arbitrary concentrations.

The free-energy changes of the reactions involving CoA were calculated with the aid of equilibrium data for the reaction

$$\text{oxaloacetate} + \text{acetyl CoA} = \text{citrate} + \text{CoA}. \quad (5)$$

According to Stern, Shapiro, Stadtman & Ochoa (1951), K_c is about 5×10^5 at pH 7·4 (i.e. $\Delta G' = -7{\cdot}8$ kcal.). The difference between this value and ΔG^0 for the condensation of oxaloacetate and acetate to form citrate (5·61 kcal.) shows that $\Delta G'$ is about 13·4 kcal. for the acetylation of CoA at pH 7·4. This is only an approximate value since neither the ΔG^0f value for acetic acid nor the equilibrium constant of reaction (5) possess the desired accuracy. Another value which is of the same order but probably no more reliable may be obtained using Stadtman's (1952) data for the reaction

$$\text{acetyl phosphate} + \text{CoA} = \text{acetyl CoA} + \text{phosphate}.$$

K_c was found to be about 60 at pH 8; this corresponds to $\Delta G' = -2{\cdot}4$ kcal. By analogy with the phosphoglyceric phosphokinase reaction (Bücher, 1947), ΔG^0 may be taken to be 4·5 kcal. for the reaction

$$\text{adenosinetriphosphate} + \text{acetate} = \text{adenosinediphosphate} + \text{acetyl phosphate}.$$

The free energy of hydrolysis of ATP to ADP is evaluated in the next section of this paper and found to be −9·4 kcal. at pH 7·5. Hence $\Delta G'$ is about −13·9 kcal. for the hydrolysis of acetyl phosphate and −11·5 kcal. for the hydrolysis of acetyl CoA at pH 7·5.

Table 5. *Free-energy data for the steps of the tricarboxylic acid cycle and certain associated reactions*

(Temp., 25°; ΔG^0 values are for unit activity. ΔG values are for 0·2 atm. O_2, 0·05 atm. CO_2, pH 7 and 0·01 M concentration of other reactants.)

Reaction	ΔG (kcal.)	ΔG^0 (kcal.)	ΔG^0 (kJ.)
$Pyruvate^- + \frac{1}{2}O_2 + CoA + H^+ \rightarrow acetyl\ CoA + CO_2$	−55	−66	−276
$Oxaloacetate^{2-} + acetyl\ CoA + H_2O \rightarrow citrate^{3-} + CoA + H^+$	−7·8	+1·8	+7·5
$Oxaloacetate^{2-} + acetate^- \rightarrow citrate^{3-}$	+8·26	+5·53	+23·14
$Citrate^{3-} \rightarrow cis\text{-}aconitate^{3-} + H_2O$	+2·04	+2·04	+8·54
$cis\text{-}Aconitate^{3-} + H_2O \rightarrow isocitrate^{3-}$	−0·45	−0·45	−1·88
$isoCitrate^{3-} + \frac{1}{2}O_2 \rightarrow oxalosuccinate^{2-} + H_2O$	−52·5	−52·95	−215·43
$Oxalosuccinate^{3-} + H^+ \rightarrow \alpha\text{-}ketoglutarate^{2-} + CO_2$	−8·6	−16·43	−68·74
$Oxalosuccinate^{3-} + H_2O \rightarrow \alpha\text{-}ketoglutarate^{2-} + HCO_3^-$	−9·5	−5·77	−35·56
$\alpha\text{-}Ketoglutarate^{2-} + \frac{1}{2}O_2 \rightarrow succinate^{2-} + CO_2$	−69·8	−68·49	−286·56
$Succinate^{2-} + \frac{1}{2}O_2 \rightarrow fumarate^{2-} + H_2O$	−35·7	−36·13	−151·17
$Fumarate^{2-} + H_2O \rightarrow malate^{2-}$	−0·88	−0·88	−3·68
$Malate^{2-} + \frac{1}{2}O_2 \rightarrow oxaloacetate^{2-} + H_2O$	−44·8	−45·24	−189·28
$\frac{1}{2}\ Butyrate^- + \frac{1}{2}O_2 + CoA + \frac{1}{2}H^+ \rightarrow acetyl\ CoA + H_2O$	−36	−42·5	−177·8
$Acetate^- + CoA + H^+ \rightarrow acetyl\ CoA + H_2O$	+16	+3·7	+15·5
$2\ Acetyl\ CoA + H_2O \rightarrow acetoacetate + 2CoA + H^+$	−13	−0·7	−2·9
$Pyruvate^- + 2\frac{1}{2}O_2 + H^+ \rightarrow 3CO_2 + 2H_2O$	−273·5	−282·84	−1183·4
$DPNH + \frac{1}{2}O_2 + H^+ \rightarrow DPN^+ + H_2O$	−51·9	−61·91	−259·03
$Glucose + 6O_2 \rightarrow 6CO_2 + 6H_2O$	−688·5	−686·32	−2871·56
$Glutamate^{+2-} + \frac{1}{2}O_2 \rightarrow \alpha\text{-}ketoglutarate^{2-} + NH_4^+$	−45·9	−43·63	−182·55
$Aspartate^{+2-} + \frac{1}{2}O_2 \rightarrow oxaloacetate^{2-} + NH_4^+$	−44·7	−42·42	−177·49
$Alanine^{+-} + \frac{1}{2}O_2 \rightarrow pyruvate^- + NH_4^+$	−45·8	−43·57	−182·30
$Glutamate^{+2-} + DPN^+ + H_2O \rightarrow \alpha\text{-}ketoglutarate^{2-} + NH_4^+ + DPNH + H^+$	+6·0	+18·28	+76·48
$Malate^{2-} + TPN^+ \rightarrow pyruvate^- + TPNH + CO_2$	−2·0	−0·18	−0·75
$Oxaloacetate^{2-} + H_2O \rightarrow pyruvate^- + HCO_3^-$	−9·12	−6·39	−26·74

The ΔG^0 value given in Table 5 (−43·63 kcal.) for the deamination of glutamate by reaction with molecular O_2 does not agree with that of −40·4 kcal. which may be calculated from the potential measurements of Filitti-Wurmser & Morel (1940). As discussed in the preceding paper (Burton & Wilson, 1953), there is often no guarantee that equilibrium has been reached in oxidation-reduction potential measurements in this type of system.

The ΔG^0 value for the reaction between glutamate and DPN corresponds to $K_a = 4{\cdot}0 \times 10^{-14}$ M². Olson & Anfinsen (1953) have obtained the value of $1{\cdot}45 \times 10^{-13}$ M² for K_c by direct measurement. It is likely that the activity coefficients account for a large part of the difference between the calculated K_a value and the experimental value for K_c.

In view of the high aconitase and fumarase activities of tissue homogenates it is interesting that Frohman *et al.* (1951) find that the tricarboxylic acids of rat tissues are present in ratios similar to those attained in the aconitase equilibrium, whereas the ratio of fumarate to malate found in the tissues (approx. 3:1) is very different to that of the fumarase equilibrium (approx. 0·2:1). This could be explained by assuming either that the fumarate and malate of the tissues are not in equilibrium or that most of the fumarate is present in a bound form.

SECTION 3

The free-energy changes associated with the hydrolysis of adenosinetriphosphate (ATP) to adenosinediphosphate (ADP)

Earlier evaluations of $\Delta G'$ for the hydrolysis of ATP to ADP and inorganic phosphate (Lipmann, 1941, 1946; Meyerhof, 1944; Dixon, 1949) have led to values which are close to −12 kcal. at pH 7·5. Despite the excellent agreement of these independent estimates, Dixon (1949), Meyerhof (1951) and Wurmser (1951) have each emphasized that some of the free-energy data on which they are based are very uncertain. Lately, Meyerhof himself has accepted a revised value of −10·5 kcal. (Oesper, 1951; Meyerhof, 1951), while Wurmser (1951) has suggested that the value of −9 kcal. is more consistent with the $\Delta G^0 f$ values for glucose and lactate and equilibrium data for the stages of glycolysis. Although Wurmser's calculation is more reliable than the others mentioned, it is still based on uncertain free-energy data, the principal inaccuracies being in the thermochemical data for lactic acid and in Meyerhof & Green's (1949) ΔG^0 value for the hydrolysis of hexosediphosphate. These inaccuracies appear almost to cancel each other so that Wurmser's

value agrees with the independent value obtained below.

Evaluation of $\Delta G'$ for the hydrolysis of ATP. Most of the equilibria considered involve multivalent electrolytes, and cumbersome equations are therefore obtained when the ionization equilibria are taken into account. These may be conveniently avoided by considering the concentrations of those ionic forms which are the major component at pH 8. At this pH other ionic forms are present in negligible amounts. Under these conditions the hydrolysis of ATP may be formulated

$$ATP^{4-} + H_2O = ADP^{-} + HPO_4^{2-} + H^+. \quad (6)$$

By adding reactions (7)–(14) listed in Table 6 the following reaction is obtained

$$\text{glycerol} + 2HPO_4^{-2} + 2ADP^{3-} + H^+ = \text{pyruvate}^- + 2ATP^{4-} + 2H_2O + 2H_2, \quad (15)$$

and the sum of the corresponding ΔG^0 values is 1·39 kcal. From this value and the $\Delta G^0 f$ values for aqueous glycerol (Table 1) and the pyruvate$^-$ ion (Table 4) the value of 1·03 kcal. is obtained for ΔG^0 of reaction (6), the hydrolysis of ATP to ADP. Since a H^+ ion appears on the right-hand side of equation (6) the relation between ΔG^0 and $\Delta G'$ (in kcal. at 25°) is

$$\Delta G' = \Delta G^0 - 1{\cdot}3643 \times \text{pH},$$

which gives the value of $-9{\cdot}25$ kcal. for $\Delta G'$ at pH 7·5. This value has been derived ignoring any possible effect of magnesium ions on the equilibria given in Table 6. Magnesium salts were added in the measurements of the phosphatase, phosphokinase, phosphoglyceric mutase and enolase equilibria (reactions 7, 12, 13 and 14) since they are required for full enzymic activity. It is necessary to consider whether the formation of complexes between the magnesium and the phosphorylated compounds is likely to affect the equilibrium measurements. This does not appear to have been tested experimentally and, unfortunately, most of the information about the formation of complexes between Mg^{2+} ions and phosphate compounds is only qualitative (Frankenthal, 1944; Neuberg & Mandl, 1949; Hers, 1952). Quantitative data based on potentiometric titration are available for inorganic phosphate; the results of Greenwald, Redish & Kibrick (1940) indicate that Mg^{2+} and HPO_4^{2-} ions form a complex, probably $MgHPO_4$, and that the dissociation constant is

$$K' = [Mg^{2+}][HPO_4^{2-}]/[MgHPO_4] = \text{antilog}_{10}(2{\cdot}15\sqrt{I} - 2{\cdot}50).$$

To obtain quantitative information for ATP and ADP, 0·004 – 0·018 M solutions of the sodium salts have been titrated with 0·5 N-HCl: the pH was measured between 8 and 2 with a glass electrode (Burton, unpublished). In the presence of increasing concentrations of $MgCl_2$, the apparent pK of the weakly ionizing phosphate group becomes more acidic while the pK of the adenine amino group is unchanged, the effect being greater with ATP than with ADP. The results are compatible with the formulation of the principal complexes as $MgATP^{2-}$ and $MgADP^-$ with dissociation constants of about $1{\cdot}2 \times 10^{-3}$ and $2{\cdot}7 \times 10^{-3}$ M respectively at an ionic strength of 0·2. Similar measurements on the Mg complex of phosphopyruvate give a dissociation constant of 10^{-2} M. For the same ionic strength the results of Greenwald *et al.* (1940) give a constant of 3×10^{-2} M for the complex of

Table 6. *Thermodynamic data for the conversion of glycerol to pyruvate*

(Addition of ΔG^0 for reactions (7)–(14) gives ΔG^0 for reaction (15). This value is used in the text to calculate ΔG^0 for the hydrolysis of ATP.)

	Reaction	K_c	ΔG^0 (kcal. at 25°)	Source
(7)	Glycerol + HPO_4^{2-} → DL-glycerol 1-phosphate^{2-} + H_2O	0·0122 (37°)	2·24	Meyerhof & Green (1949)
(8)	DL-Glycerol 1-phosphate^{2-} → L-glycerol 1-phosphate^{2-}	—	0·41	Calculated (= RT ln 2)
(9)	L-Glycerol 1-phosphate^{2-} → dihydroxyacetone phosphate^{2-} + H_2	—	10·24	Burton & Wilson (1953)
(10)	Dihydroxyacetone phosphate^{2-} → glyceraldehyde 3-phosphate^{2-}	0·045	1·83	Meyerhof & Junowicz-Kocholaty (1943)
(11)	Glyceraldehyde 3-phosphate^{2-} + HPO_4^{2-} → glyceroylphosphate 3-phosphate^{4-} + H_2	—	5·83	From data of Meyerhof & Oesper (1947) see Burton & Wilson (1953)
(12)	Glyceroylphosphate 3-phosphate^{4-} + ADP^{3-} → glycerate 3-phosphate^{3-} + ATP^{4-}	3200	−4·78	Bücher (1947)
(13)	Glycerate 3-phosphate^{3-} → enolpyruvate 2-phosphate^{3-} + H_2O	—	0·42	Meyerhof & Oesper (1949)
(14)	Enolpyruvate 2-phosphate^{3-} + ADP^{3-} + H^+ → pyruvate$^-$ + ATP^{4-}	$7{\cdot}1 \times 10^{40}$	−14·80	
(15)	Glycerol + $2HPO_4^{2-}$ + $2ADP^{3-}$ + H^+ → pyruvate$^-$ + $2ATP^{4-}$ + $2H_2O$ + $2H_2$	—	1·39	—

inorganic phosphate. For present purposes it is assumed that phosphate compounds not containing the pyrophosphate group possess an affinity for Mg^{2+} ions similar to that of phosphopyruvate. The above dissociation constants then indicate that, in Bücher's (1947) measurements of the phosphoglyceric phosphokinase equilibrium, 10–15% of the ATP and 5–10% of the ADP were present as Mg complexes. In the case of the pyruvate phosphokinase equilibrium (Meyerhof & Oesper, 1949) it appears that about 30% of the ATP and 20% of the ADP were present as complexes. The corrections thus introduced into the ΔG^0 values for these reactions are very small and correspond to an increase of about 0·1 kcal. in $\Delta G'$ for the hydrolysis of ATP. The value of 9·4 kcal. at pH 7·5 is therefore adopted for subsequent calculations.

The largest single source of error in this value is probably ΔG^0 for the pyruvate phosphokinase reaction (14) since the pH was not reported in the only equilibrium measurements which are available (Meyerhof & Oesper, 1949). In a private communication Dr Meyerhof has stated that the pH was probably about 7·6. The data for the phosphoglyceric mutase and enolase equilibria (13) may introduce a small additional error because the equilibrium constant obtained in the presence of crystalline enolase (Warburg & Christian, 1942) does not agree with that calculated by Meyerhof & Oesper (1949) from the equilibrium in the presence of a crude enzyme system containing phosphoglyceric mutase together with data for the phosphoglyceric mutase equilibrium. The discrepancy amounts to a factor of 2·05 in the enolase equilibrium (0·43 kcal.). The values of Meyerhof & Oesper have been adopted in Table 6 because they are supported by earlier measurements (Lohmann & Meyerhof, 1934; Meyerhof & Kiessling, 1935; Akano, 1935). The equilibrium data for the other reactions in Table 6 are based on extensive measurements mainly with purified enzyme systems, and in most instances they have been confirmed by independent investigators. It should be pointed out that only half of any error in one of these ΔG^0 values is introduced into the value of $\Delta G'$ for the hydrolysis of ATP.

The ΔG^0 values for the dehydrogenation of glycerol phosphate and triosephosphate were calculated from data at low ionic strengths where the activity coefficients are presumably close to unity. Activity data are not available for the other reactions of Table 6 but, with the possible exception of the pyruvate phosphokinase reaction, it is probable that the activity coefficients of substances on opposite sides of each reaction will largely cancel each other.

The value of $-9{\cdot}4$ kcal. for the free energy of hydrolysis of ATP at pH 7·5 refers to thermodynamic standard conditions (i.e. molal concentrations of ATP, ADP and inorganic phosphate). At the concentrations usually present in tissue homogenate experiments and in intact cells the numerical value of ΔG is greater. The relation between ΔG (in kcal. at 25°) and ΔG^0 is as follows

$$\Delta G = \Delta G^0 - 1{\cdot}3643 \times \left\{ \mathrm{pH} + \log_{10} \frac{[\mathrm{ATP}^{4-}]}{[\mathrm{ADP}^{3-}][\mathrm{HPO_4}^{2-}]} \right\}.$$

Between pH 7·5 and 11 the concentration of each of these ions is practically equal to the total concentration of the respective compound. There is therefore in this range a linear relationship between ΔG and pH for constant concentrations of ATP, ADP and inorganic phosphate, although in more acid solutions other ionic forms may predominate with the result that, as discussed by Dixon (1949) and by Alberty, Smith & Bock (1951) ΔG becomes practically independent of the pH below pH 6·3.

Homogenate experiments. The concentrations used in the homogenate experiments of Krebs, Ruffo, Johnson, Eggleston & Hems (1953) are of the same order as those in many previous studies of oxidative phosphorylation and are as follows: 5×10^{-3}M-inorganic phosphate, $2{\cdot}5 \times 10^{-3}$M-ATP, about 2×10^{-4}M-ADP and $2{\cdot}5 \times 10^{-3}$M-$MgCl_2$ at pH 7·4. Under these conditions ΔG is calculated to be -14 kcal. ignoring the complex-forming effect of the Mg^{2+} ion and $-13{\cdot}8$ kcal. when this is taken into account.

Animal tissues. ΔG may be calculated for the concentrations of ATP, ADP and inorganic phosphate reported to be present in animal tissues. Taking the pH to be 7, the data of LePage (1946) for rat tissues (as given by Dickens, 1951) give values for ΔG which are between $-12{\cdot}5$ and -16 kcal. neglecting any complex formation between magnesium and the phosphate compounds. If the intracellular pH is 6·3 the values would be about 0·5 kcal. more positive. These are only rough estimates of the free energy changes since they are calculated from relatively few analyses on the assumption that the ATP, ADP and inorganic phosphate are uniformly distributed throughout the tissues. It might be thought that the tissue magnesium (3–4 μmoles/g.) could as a result of complex formation invalidate these results, but since all three reactants are likely to form complexes the corrections to be applied for this effect amount to less than 0·3 kcal. in the ΔG values.

Hydrolysis of ADP and creatinephosphate. Other high-energy phosphate compounds in tissues are likely to be almost in equilibrium with ATP and ADP as the activities of the phosphokinases are relatively high. Hence the ΔG values for the hydrolysis of creatinephosphate and the terminal phosphate group of ADP in tissues are likely to be close to that for the hydrolysis of the terminal phosphate group of ATP even if the free-energy changes at standard concentrations ($\Delta G'$) may not be the same.

Measurements of the myokinase equilibrium (Eggleston & Hems, 1952) show that slightly more free energy is liberated by the hydrolysis of ADP to AMP under standard conditions than by the hydrolysis of ATP to ADP. The difference amounts to 0·48 kcal. ignoring the effect of Mg^{2+} ions on the equilibrium; it is expected to be 0·6–0·8 kcal. if the Mg^{2+} ion effect is taken into account.

Banga's (1943) measurements of the creatine phosphokinase equilibrium when combined with the

above value of $\Delta G'$ for the hydrolysis of ATP indicate that ΔG^0 is about $-12{\cdot}8$ kcal. for the hydrolysis of creatinephosphate in the pH range between 7·5 and 11

$$\text{creatinephosphate}^{+2-} + H_2O = \text{creatine}^{+} + HPO_4^{2-}.$$

SECTION 4

The free-energy changes associated with the individual steps of glycolysis and alcoholic fermentation

Equilibrium measurements (including those referred to in Table 6) are available for all the individual enzyme-catalysed reactions of glycolysis except the glucokinase and phosphofructokinase reactions. The ΔG^0 values for these reactions may be calculated by combining the value for the hydrolysis of ATP with those for the hydrolysis of the hexose phosphates. Although the thermochemical data for starch and glycogen are inadequate, $\Delta G^0 f$ for the glucose units of these polysaccharides may be evaluated using data for glucose 6-phosphate and for the phosphoglucomutase and phosphorylase reactions.

ΔG^0 for the hydrolysis of hexose phosphates. Meyerhof & Green (1949) have measured the equilibria between several sugar phosphates, the free sugar and inorganic phosphate in the presence of purified intestinal phosphatase. The equilibria are very much in favour of hydrolysis and high concentrations of the sugar and of inorganic phosphate were necessary. Inadequate data are available to evaluate the thermodynamic activities under these conditions so that the ΔG^0 values are not as reliable as most values derived from equilibrium measurements. The ΔG^0 values for glucose 6-phosphate (G 6-P) and fructose 6-phosphate (F 6-P) are $-3{\cdot}0$ and $-3{\cdot}3$ kcal. respectively. Slein's (1950) measurements of the phosphoglucomutase equilibrium give the value of 0·50 kcal. for ΔG^0 of the conversion of G 6-P to F 6-P and Wolfrom & Lewis (1928) found that, in the epimerization of glucose, the ratio of fructose to glucose became constant when it reached 0·49; if this ratio represents the equilibrium between glucose and fructose, ΔG^0 is 0·44 kcal. The difference of 0·06 kcal. between the ΔG^0 value for the epimerization of glucose and that for the phosphoglucomutase reaction should equal the difference between the ΔG^0 value for the hydrolysis of F 6-P and that for G 6-P. There is therefore a small discrepancy in these data, but the evidence is inadequate to decide whether Meyerhof & Green's (1949) values are at fault or whether the ΔG^0 value for the epimerization is incorrect and somewhat arbitrary values must therefore be adopted. In this paper the ΔG^0 values of $-3{\cdot}1$ and $-3{\cdot}2$ kcal. are used for the hydrolysis of G 6-P and F 6-P respectively.

Meyerhof & Green's ΔG^0 values for the hydrolysis of fructose 1:6-diphosphate (HDP) cannot be accepted on the present evidence since the F 6-P and fructose 1-phosphate (F 1-P) in the equilibrium mixture probably interfered in the estimation of HDP by conversion to triosephosphate under the action of aldolase. The available evidence indicates that aldolase is not specific to HDP and may act on F 6-P (Dounce, Barnett & Beyer 1950), and possibly also on F 1-P since Meyerhof, Lohmann & Schuster (1936) have shown that dihydroxyacetone phosphate may condense with D-glyceraldehyde to form F 1-P, and it is therefore likely that the reverse reaction can occur. The following alternative procedure for the evaluation of ΔG^0 for the hydrolysis of HDP gives a value ($-7{\cdot}6$ kcal.) which differs from that of Meyerhof & Green ($-4{\cdot}8$ kcal.) in the expected direction.

From Meyerhof & Lohmann's (1934) data for the equilibrium between dihydroxyacetone phosphate and HDP under the action of aldolase and isomerase, ΔG^0 is $-3{\cdot}68$ kcal. at 25°. The reaction is formulated

$$2 \text{ dihydroxyacetone phosphate}^{2-} = HDP^{4-},$$

and on adding to it twice the sum of the reactions (7)–(9) of Table 6, the following reaction is obtained

$$2 \text{ glycerol} + 2HPO_4^{2-} = HDP^{4-} + 2H_2O + 2H_2. \quad (17)$$

Addition of the corresponding ΔG^0 values gives the value of 22·10 kcal. for reaction (17). From this value, the ΔG^0 value for the conversion of glucose to fructose (0·44 kcal. from Wolfrom & Lewis's data), and from the $\Delta G^0 f$ values for glucose and glycerol (Table 1) ΔG^0 is found to be $-7{\cdot}6$ kcal. for the reaction

$$HDP^{4-} + 2H_2O = \text{fructose} + 2HPO_4^{2-}.$$

Starch and glycogen. In the synthesis or degradation of either starch or glycogen under the influence of the appropriate phosphorylase, the reaction ceases when the ratio of the concentrations $[\text{G 1-P}^{2-}]/[HPO_4^{2-}]$ becomes 0·40 (Hanes & Maskell, 1942; Cori, Cori & Green, 1943). The reaction may be formulated

$$(\text{glucose})_n + \text{G 1-P}^{2-} = (\text{glucose})_{n+1} + HPO_4^{2-}.$$

The equilibrium ratio of G 1-P to inorganic phosphate is independent of the polysaccharide concentration. This is expected if, as is likely, all the glucose units of the polysaccharide which react in the phosphorylase system are associated with the same free energy. ΔG^0 for the hydrolysis of G 1-P ($-4{\cdot}8$ kcal.) may be obtained from the value for G 6-P and the phosphoglucomutase equilibrium (Colowick & Sutherland, 1942). The free-energy change for the hydrolysis of a glycosidic link of starch or glycogen is therefore $-4{\cdot}8 - RT \ln 0{\cdot}40 = -4{\cdot}3$ kcal. This, together with the ΔG^0 value for glucose, gives a $\Delta G^0 f$ value of $-158{\cdot}4$ kcal. for the glucose units ($C_6H_{10}O_5$) of the polysaccharides.

It should be emphasized that this value refers only to those glucose units which react in the phosphorylase system and, like the equilibrium of that system, is independent of the polysaccharide concentration.

The individual reactions of glycolysis and alcoholic fermentation. The free-energy data for the stages of these processes (Table 7) are based on the $\Delta G^0 f$ values in Table 1 for glucose, ethanol, acetaldehyde and carbon dioxide, on data for the hydrolysis of ATP to ADP, on some data given in Table 6, and on the equilibrium measurements of Racker (1950) for the lactic and alcohol dehydrogenase systems, together with the data already presented in this section.

It will be seen that the conversion of glycogen to lactate liberates about 28 kcal./mole lactate$^-$ ion at pH 7. According to the accepted mechanism of glycolysis (cf. Dickens, 1951) this is accompanied by

Table 7. *Free-energy data for the steps of glycolysis and alcoholic fermentation*

(Temp., 25°; ΔG^0 values are for unit activity, ΔG values are for 0·2 atm. O_2; 0·05 atm. CO_2; pH 7 and 0·01 M concentration of other reactants except glycogen. As discussed in the text, the data for the reactions involving glycogen are independent of the concentration of glycogen.)

Reaction	ΔG (kcal.)	ΔG^0 (kcal.)	ΔG^0 kJ.
Glycogen (1 glucose unit) + H_2O → 2 lactate$^-$ + $2H^+$	−56·7	−32·2	−134·72
Glucose → 2 lactate$^-$ + $2H^+$	−49·7	−27·9	−116·73
Glucose → 2 ethanol + $2CO_2$	−62·2	−55·9	−233·89
Glucose + ATP^{4-} → G 6-P^{2-} + ADP^{3-} + H^+	−5·7	+3·9	+16·32
G 6-P^{2-} → G 1-P^{2-}	+1·72	+1·72	+7·20
G 1-P^{2-} + H_2O → glycogen + HPO_4^{2-}	−0·55	−0·55	−2·30
G 6-P^{2-} → F 6-P^{2-}	+0·50	+0·50	+2·09
F 6-P^{2-} + ATP^{4-} → HDP^{4-} + ADP^{3-} + H^+	−4·3	+5·2	+21·76
HDP^{4-} → glyceraldehyde 3-P^{2-} + dihydroxyacetone P^{2-}	+2·78	+5·51	+23·05
Dihydroxyacetone-P^{2-} → glyceraldehyde 3-P^{2-}	+1·83	+1·83	+7·66
Glyceraldehyde 3-P^{2-} + DPN^+ + HPO_4^{2-} → glyceroyl-P 3-P^{4-} + DPNH + H^+	+4·2	+11·05	+46·23
Glyceroyl-P 3-P^{4-} + ADP^{3-} → glycerate 3-P^{3-} + ATP^{4-}	−4·75	−4·75	−19·87
Glycerate 3-P^{3-} → glycerate 2-P^{3-}	+1·06	+1·06	+4·44
Glycerate 2-P^{3-} → enolpyruvate 2-P^{3-} + H_2O	−0·64	−0·64	−2·68
Enolpyruvate 2-P^{3-} + ADP^{3-} + H^+ → pyruvate$^-$ + ATP^{4-}	−5·0	−14·5	−60·67
Pyruvate$^-$ + DPNH + H^+ → lactate$^-$ + DPN^+	−6·0	−15·54	−65·02
Pyruvate$^-$ + H^+ → acetaldehyde + CO_2	−6·9	−14·65	−61·30
Acetaldehyde + DPNH + H^+ → ethanol + DPN^+	−5·4	−14·90	−62·34
ATP^{4-} + H_2O → ADP^{3-} + HPO_4^{2-} + H^+	−11·3	+0·9	+3·77

a net formation of 1·5 moles high-energy phosphate (~P) per mole of lactate formed. Assuming 14 kcal. per mole ~P this would require 21 kcal. or 75 % of the total free energy available. If glucose is the starting material, about 25 kcal. are available per mole of lactate, but the net amount of ~P formed is 1 mole per mole lactate, which corresponds to about 56 % of the total free energy available. The free energy available from the alcoholic fermentation of glucose (31 kcal./mole ethanol) is appreciably greater than that from the lactic fermentation; the formation of 1 ~P per mole ethanol corresponding to 14 kcal. represents 44 % of the available energy.

SUMMARY

1. The free-energy changes for the individual stages of the main biological energy-giving reactions have been re-evaluated with improved accuracy.

2. Data for the heats of combustion and entropies of compounds of biological interest have been critically selected and used to evaluate the free energies of formation ($\Delta G^0 f$) which are listed in Table 1.

3. From values contained in Table 1 and equilibrium data for reactions associated with the tricarboxylic acid cycle values of $\Delta G^0 f$ (listed in Table 4) have been obtained for several compounds which are intermediates of, or associated with, the cycle. The free-energy changes for the stages of the tricarboxylic acid cycle and for certain associated reactions are given in Table 5 and those for the stages of glycolysis and alcoholic fermentation are given in Table 7.

4. The free-energy change associated with the hydrolysis of adenosinetriphosphate (ATP) to adenosinediphosphate (ADP) and inorganic phosphate has been evaluated from the values of $\Delta G^0 f$ for glycerol and pyruvate and from the equilibrium data for the enzymic stages of the conversion of glycerol to triosephosphate, and subsequently to pyruvate, coupled with the phosphorylation of ADP. The value obtained is −9·4 kcal. for pH 7·5 and standard concentrations (1 M activity) of ATP, ADP and inorganic phosphate; for the concentrations in animal tissues it is 13–16 kcal.

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Oxidative Phosphorylation

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It is known from the work of Engelhardt (1930, 1932), Runnström, Lennerstrand & Borei (1934), Kalckar (1939, 1941, 1944), Belitzer & Tsibakowa (1939), Cori and his associates (Colowick, Welch & Cori, 1940; Colowick, Kalckar & Cori, 1941) and Ochoa (1941, 1943, 1947) that the free energy of oxidative processes can be used for the synthesis of organic phosphates, including those containing energy-rich bonds, namely, adenosinetriphosphate (ATP) or creatine phosphate. The quantitative aspects of the 'oxidative phosphorylation' i.e. the value of the ratio

$$\frac{\text{equivalents of organic phosphate formed}}{\text{atoms of oxygen consumed}}$$

has been studied by Ochoa (1941, 1943) and more recently by Hunter & Hixon (1949*a*, *b*), Lehninger & Smith (1949), Green, Atchley, Nordmann & Teply (1949), Pardee & Potter (1949), Slater (1950), Judah & Williams-Ashman (1951) and others. The values obtained for the above ratio, henceforth in this paper called 'phosphorylation quotient', depend on experimental conditions. Of special interest are the maximum values that can be obtained. The highest quotients found so far approach the value of 4 (Barkulis & Lehninger, 1951*a*; Judah, 1951). These values were usually obtained with tissue preparations which contained, apart from the oxidizing enzymes, ATP, hexokinase, inorganic phosphate and fluoride, and under these conditions most of the inorganic phosphate utilized appears as hexosephosphate. The presence of fluoride is required to prevent dephosphorylation of organic phosphate. The phosphate uptake values measured by this procedure have to be regarded as minimum values as dephosphorylation may not be completely abolished.

The present paper is concerned with measurements of the phosphorylation quotient by a procedure employing ^{32}P which has the advantage of being independent of the action of dephosphorylating enzymes. The use of ^{32}P as a tracer, combined with newer methods of separating the phosphorylated compounds by paper chromatography, makes it possible to measure directly the rate of synthesis of ATP even when no changes in the concentrations of ATP or of inorganic phosphate occur. It is therefore unnecessary to add fluoride or to apply corrections.

EXPERIMENTAL

A respiring tissue preparation, reinforced with oxidizable substrates, ATP and ^{32}P in the form of inorganic orthophosphate (hereafter referred to as $^{32}PO_4$) is shaken in the presence of O_2, the conditions being such as to keep the rate of respiration and the concentration of ATP and inorganic phosphate approximately constant. The rate of oxygen absorption is measured manometrically and the rate of formation of energy-rich phosphate bonds is calculated from the rate at which $^{32}PO_4$ is incorporated into the ATP molecule. The procedure requires specific activity determinations of the phosphate fractions in the tissue preparations, which in turn involve separation and quantitative determination of the various phosphates. Hanes & Isherwood (1949) have supplied the chromatographic methods required for these separations.

It is already qualitatively known (Hummel & Lindberg, 1949) that ATP exchanges phosphate groups with inorganic phosphate very rapidly. In liver homogenates containing about 10% (fresh weight) tissue and 5×10^{-3} M labile ATP phosphate the turnover rate of ATP (i.e. the time required to exchange a quantity of phosphate equal to the labile phosphate of ATP) is of the order of minutes at body temperature. This is too rapid for precise measurements. Experiments must therefore be carried out with dilute tissue suspensions (3%) at a low temperature.

Measurement of turnover rate of ATP. It is assumed that two substances, M and N, combine reversibly to form MN.

$$M + N \rightleftharpoons MN,$$

further, that a steady state is maintained so that the concentrations of the reactants remain constant and that the three reactants do not take part in other processes. No assumption is required about the mechanism maintaining the steady state. It is also irrelevant whether the 'reversible' reaction is in thermodynamic equilibrium or whether it is (as in the case here considered) a cyclic system in which the forward and backward reactions involve different mechanisms and different intermediate stages. If an isotopic form of M (or N) is introduced and the rate of its disappearance or of its incorporation into MN is measured, the rates of the forward and backward reactions of the system can be calculated; the problem, as Sheppard (1948) has pointed out, is identical with that of calculating the exchange of inorganic ions between tissue and surrounding mediums. The following formula of Krebs, Eggleston & Terner (1951) is applicable:

$$v = \frac{ab}{t(a+b)} \ln \frac{bx_o}{x(a+b) - ax_o}, \qquad (1)$$

where v = quantity of N converted into, and formed from MN, per unit time; a = quantity of N; b = quantity of MN; x_o, x = relative quantity (specific activity) of isotope form of N at t_o, t.

In applying this equation to the phosphorylation of ATP it has to be borne in mind that two phosphate groups of ATP may be readily interconvertible with inorganic phosphate. The position is therefore described by the scheme

$$M + 2N \rightleftharpoons MNN, \qquad (2)$$

where M represents adenosinemonophosphate (AMP), N inorganic phosphate and MNN adenosinetriphosphate. At least two stages are involved in the formation of MNN, one N being added or lost at each step. The work on myokinase (Kalckar, 1943) suggests that the following two reactions are involved (ADP = adenosinediphosphate):

$$\left.\begin{array}{l} \text{ADP} + \text{phosphate} \rightleftharpoons \text{ATP}, \\ \text{AMP} + \text{ATP} \rightleftharpoons 2\text{ADP}. \end{array}\right\} \qquad (3)$$

But, whatever the mechanism, the mathematical analysis (see Appendix) shows that formula (1) is also applicable to the reactions (2).

In using the formula (1) for the system (2), a represents the quantity of inorganic phosphate and b the quantity of labile ATP phosphorus. If a is expressed in μmoles, b has to be given in μmoles of labile ATP phosphorus, i.e. two-thirds of the total ATP phosphorus.

Experimental arrangements. To avoid complications from permeability barriers and slow diffusion, tissue suspensions (homogenates) were used. As already mentioned, the rates of O_2 uptake and of oxidative phosphorylation in liver, heart or kidney suspensions are very rapid at 40°; even after 30-fold dilution of the tissue, added ATP was found to come into equilibrium with $^{32}PO_4$ within a few minutes, a rate at which no exact measurements of pairs of values for x and t can be made. Since dilution beyond 30-fold is liable to cause loss of enzyme activity, the reaction rate was reduced by lowering the temperature to 20°.

Special experimental arrangements are required so that the two premises hold true on which the theroretical treatment of the experimental finding rests, namely (1) that adenosine polyphosphates and inorganic phosphate are the only phosphorus compounds metabolized in the system; (2) that the concentrations of both ATP and inorganic phosphate remain constant. Both assumptions can never be rigidly correct but, by suitable choice of experimental conditions, the deviations from the premises can be made so small that they may be neglected.

Dilution of the tissue reduces the concentration of endogenous phosphates, and if the concentrations of added ATP and inorganic phosphate are relatively high ($0{\cdot}5 \times 10^{-2}$ M) the concentrations of all other phosphates which react metabolically are small. Moreover, the reactions between ATP and inorganic phosphate are relatively rapid in comparison with the reactions of other phosphates (e.g. the synthesis of nucleic acids or the phosphorylation of glucose through the hexokinase reaction) and side reactions are therefore in any case of negligible proportions (a few % in 15 min.). Their precise extent is measured in every experiment by the determination of the $^{32}PO_4$ in compounds other than ATP and inorganic phosphate.

The validity of assumption (2) is directly checked for each experiment by the quantitative determination of ATP and of inorganic phosphate in the system.

Special chemicals. Three different preparations of ATP were used. The first, used in about four-fifths of all experiments, was a barium salt prepared from rabbit muscle according to the directions of LePage (1949); the second was a commercial barium salt supplied by Boots Pure Drug Co. Ltd. and purified by dissolving in 0·2 N-HNO_3 and reprecipitation with Hg and Ba as described by LePage; the third was a preparation of the free acid kindly given by Roche Products Ltd. To convert the barium salts into the sodium salts, 85·5 mg. were dissolved in 0·8 ml. N-HCl and water was added to 4·2 ml. The Ba was precipitated by the calculated amount of Na_2SO_4 (0·4 ml. 0·5 M), the precipitate was centrifuged off and the supernatant was neutralized, with about 0·4 ml. 2 N-NaOH. All operations were carried

Table 1. *Composition of samples of ATP*

Nature of preparation	Percentage distribution of total phosphate					
				Inorganic		
	ATP	ADP	AMP	Ortho-phosphate	Pyro-phosphate	Other phosphates
Free acid, Roche Products Ltd.	68·8	21·8	3·7	0	0	5·7
Barium salt prepared according to LePage (1949)	86·0	11·0	0	3·0	0	0

out at 0°. The concentration of ATP in the solution was about 0·016 M, i.e. 80 % of the expected, owing to the loss by adsorption on to the precipitate (Bailey, 1949).

The concentration and purity of the ATP solution was determined by paper chromatography, by the method of Eggleston & Hems (1952) and by phosphate determination in the solutions. Representative results are shown in Table 1. None of the preparations tested was 100 % ATP. The preparation used in most experiments contained 86 % ATP and 11 % ADP.

Carrier-free $KH_2{}^{32}PO_4$ prepared from ^{32}S by bombardment with neutrons was obtained in aqueous solution from the Atomic Energy Research Establishment at Harwell. The sample, as received, was dissolved in about 0·5 ml. water and had an activity of about 1 mc. This solution was diluted with 'stock saline' to 10 ml. and a measured quantity of this dilution, giving about 1×10^6 counts/min. was used per vessel. When freshly prepared, 0·1 ml. solution was required and this volume was measured with the Agla syringe of Burroughs Wellcome Ltd. For the sake of convenience and rapidity the tip of the syringe was fitted with a 15 cm. length of flexible plastic Portex drainage tubing no. 4 (internal diam. 2 mm.) provided with a tip of the same type of tubing no. 1 (1 mm. diam.). During the delivery of the radioactive solution into the manometer cups some slight pressure on the tubing, causing errors in the amounts delivered of 3–4 %, was unavoidable. The effects of such errors were eliminated in the final calculation by using the % of total radioactivity present in the various phosphate fractions. The quantity of $^{32}PO_4$ added to the saline did not appreciably affect the total phosphate concentration. The radioactivity of the incubated tissue preparation was thus about 2–3 μc./ml. solution.

Tissue suspensions. In deciding on the composition of the medium, the main consideration was to maintain respiration and to make the level of inorganic phosphate of the same order as that of labile ATP phosphates, as approximate equality of inorganic phosphate and ATP favour the accuracy of measurement. Tissue (3 g.) from a freshly killed animal was ground with 60·7 ml. 'stock saline', made up of the medium III described by Krebs (1950) (omitting $CaCl_2$, phosphate and organic compounds), 2·8 ml. 0·1 M-sodium phosphate buffer, pH 7·4, and 1·0 ml. 0·1 M-$MgCl_2$. All saline media were ice-cooled, and grinding was carried out in an ice-cooled stainless-steel homogenizer similar to the glass apparatus recommended by Potter & Elvehjem (1936). The final pH of the suspension was 6·6–6·8. The incubation and measurement of the O_2 uptake was carried out in conical Warburg vessels, which were placed in ice while they were prepared for the experiment. The main compartment contained 3 ml. of the tissue suspension, the side arm contained 1 ml. of the substrate solution including ATP, $^{32}PO_4$ and oxidizable substrates. All substrates were added as neutral sodium salts. The gas space contained O_2.

When the manometers were ready for incubation the contents of side arm and main compartment were thoroughly mixed and the manometer was attached to the water bath kept at 20°. At intervals manometers were removed from the bath and the cups were transferred as rapidly as possible to a dish containing iced water. To stop further enzyme activity 0·5 ml. 30 % trichloroacetic acid (TCA) (w/v) was added. The contents of the cup were then centrifuged for 15 min. at 0° and the supernatant was stored at −14° until it could be analysed. A number of duplicate sets without isotope were incubated at the same time in order to have sufficient material for the quantitative determination of non-isotopic substrates. In these cups 1 ml. 2 M-HCl was used to stop enzyme activity, because the yields of succinate and α-ketoglutarate were slightly low in the presence of TCA.

Measurement of O_2 uptake. As it was not possible, because of thermal equilibration, to measure manometrically the O_2 uptake for the first few minutes, measurements were made on at least three parallel manometers incubated for longer periods and the average rates were used in the calculation. The rate of O_2 uptake usually remained constant for at least 40 min. and the subsequent fall was only slight. Hence extrapolation for the initial period was sufficiently accurate.

Separation of phosphate fractions. By the addition of suitable quantities of inorganic orthophosphate and ATP the experimental conditions were so arranged that these two phosphate fractions formed about 94 % of all phosphates in the TCA filtrate. The remaining 6 % came partly from the tissue, partly from impurities in the ATP preparations. Adenosine phosphates were separated from inorganic phosphate and other organic phosphates by the paper-chromatographic methods of Hanes & Isherwood (1949) and Eggleston & Hems (1952).

Measurement of radioactivity. The beta counter tube (liquid type) of Veall (1948) was used. All the samples counted contained approximately 1·5 N-H_2SO_4 (final concentration) as they were diluted after ashing without neutralization. Care was required to avoid adherence of $^{32}PO_4$ to the counter tube. This was found to occur when the radioactive solutions were neutral or alkaline. Two washings of the tube with N-H_2SO_4 followed by washings in water were found to remove any adherent $^{32}PO_4$, whereas washing with KH_2PO_4 or K_2HPO_4 was ineffective.

Calculation of labile adenosinepolyphosphate P. The initial amounts of labile adenosinepolyphosphate P were known from the analyses for ADP and ATP of the starting material. In incubated material only the sum of ATP and ADP was determined in most experiments, chromatography with the formic acid-*iso*propyl ether solvent being used. In general, only minor changes occurred in the sum of ATP and ADP during incubation, usually within the limits of error, but corrections were nevertheless introduced on the assumption that any decrease in the sum of ATP + ADP was solely due to a conversion of ATP into ADP. This assumption rests on

the observation that the ultraviolet photographic technique of Markham & Smith (1949) did not reveal at any time measurable amounts of AMP. The method would have shown quantities of AMP amounting to 3% (in terms of molecular equivalents) of those of ATP + ADP. The formation of AMP could therefore be neglected.

Differential determination of the specific activities of the two labile phosphate groups of ATP. The principle used was as follows. ATP was hydrolysed enzymically to ADP and inorganic phosphate and the two products were separated by paper chromatography. The specific activity of the ADP-phosphate was never found to be more than half the specific activity of inorganic phosphate, which means that under the test conditions only one phosphate of ADP reacted. Hence it was unnecessary to separate the phosphates of ADP, as all radioactivity found could be attributed to the terminal phosphate of ADP.

The TCA extract of the suspension (1 ml.) was carefully neutralized with 2 N-NaOH solution from a microburette, with a trace of phenol red as indicator. After buffering with 0·2 ml. 0·1 M-triethanolamine buffer (pH 7·4) 0·1 ml. of the soluble ATP-ase preparation of Kielley & Meyerhof (1948) was added and the volume was adjusted to 1·5 ml. The solution was incubated for 30 min. at 38°, which proved sufficient for the complete hydrolysis of ATP. Chromatography was carried out according to Eggleston & Hems (1952) on 15–25 μl. samples.

Quantitative determination of substrates. As far as practicable the amounts of substrate removed and of end products formed were determined with incubated suspensions. The determinations were carried out, after centrifugation, in the supernatant of the HCl-acidified suspensions. Deproteinization was unnecessary except in the determination of citric acid and of the sum of α-ketoglutaric and succinic acids. Succinic acid was determined manometrically in the presence of succinic dehydrogenase (Krebs, 1937) without ether extraction, as interfering substances were not present. The sum of fumaric and malic acids was determined manometrically according to Nossal (1952) with washed suspensions of *Lactobacillus arabinosus*. Separate values for the two dicarboxylic acids were not required. α-Ketoglutaric acid was determined manometrically according to Krebs (1951) and the sum of α-ketoglutaric and succinic acid according to Krebs & Eggleston (1945). Citric acid was determined according to Weil-Malherbe & Bone (1949).

Control of substrate changes. Attempts were made to assess the contributions of individual oxidative reactions to the uptake of phosphate. It has not proved possible to separate quantitatively the individual enzyme systems without interfering with the phosphorylation mechanisms, but some degree of separation was achieved by the addition of intermediates in relatively high concentrations. When 0·01 or 0·02 M-succinate was added, the main substrate change was the conversion of succinate into fumarate and malate, the amounts of O_2 used, succinate removed and fumarate plus malate formed being roughly in the proportions 1:2:2 (see Table 2). When 0·01 or 0·02 M-α-ketoglutarate was the substrate, its conversion into fumarate and malate was the main, though by no means sole reaction. In Exp. 2, Table 3, for example, the amounts of O_2 used, α-ketoglutarate removed, and fumarate plus malate formed were in the proportion 1:0·78:0·64, instead of 1:1:1 expected for the quantitative reaction: α-ketoglutarate→fumarate + malate. Malonate

Table 2. *Incorporation of $^{32}PO_4$ into ATP in the presence of succinate*

(Rat-liver suspension, 1:30; succinate, 0·02 M; temp., 20°. All data refer to 4 ml. suspension. Substrate changes after 60 min.; succinate removed, 1104 μl.; fumarate and malate formed, 920 μl.)

Period of incubation (min.)	0	1	2	3	4	6	8	10	20	30	60
Total labile phosphate of ATP + ADP (μmoles)	18·4	17·7	18·4	18·4	19·1	18·7	17·5	17·9	17·9	17·2	17·5
Specific activity of labile phosphate (counts/min./μmole)	0	4 560	8 560	11 670	12 700	15 170	17 880	19 300	22 300	23 950	24 200
Inorganic phosphate (μmole)	18·8	18·8	18·8	18·3	18·3	18·3	18·9	17·9	17·0	16·9	16·9
Specific activity of inorganic phosphate (counts/min./μmole)	52 800	46 400	40 750	39 200	36 750	34 800	31 650	32 100	30 200	28 400	25 000
Ratio: specific activity of labile phosphate / specific activity of inorganic phosphate	—	0·098	0·210	0·298	0·346	0·435	0·564	0·600	0·739	0·844	0·969
Distribution of radioactivity: (as % of total activity):											
inorganic phosphate	100·0	91·5	82·6	77·0	73·3	69·1	65·6	61·9	55·0	52·2	47·3
ATP + ADP	0	8·4	17·4	23·0	26·0	30·4	33·8	36·7	42·0	44·1	46·7
other phosphate	0	0·1	0	0	0·7	0·5	0·6	1·4	3·0	3·7	6·0
v (rate of exchange of labile phosphate (in μmoles/min.))	—	1·75	2·00	1·88	1·74	1·45	1·42	1·28	1·00	0·84	—
O_2 uptake with succinate (μl.)	—	9·8	19·6	29·4	39·2	58·8	78·4	98	196	294	555
O_2 uptake without succinate (μl.)	—	2·2	4·4	6·6	9·0	13·2	18·0	22·5	45	67	118
Ratio: labile phosphate exchanged / oxygen absorbed	—	2·00	2·29	2·15	1·99	1·66	1·62	1·46	1·14	0·96	—

proved unsuitable for the separation of the step α-ketoglutarate → succinate because ATP was not maintained in the presence of 0·002 M-malonate.

RESULTS

Rate of incorporation of inorganic phosphate into ATP

Succinate as substrate. Details of a representative experiment are given in Table 2. The amounts of ATP + ADP and of inorganic phosphate remained virtually constant throughout the 60 min. period of incubation. Thus a steady state existed with regard to these two substances, as was postulated in the theoretical treatment. At the same time the specific activities of both substances underwent a change, inorganic phosphate gradually losing its activity and ATP becoming increasingly active. At the end of the incubation these two compounds still accounted for 94 % of the total radioactivity in the system. It follows that no major side reactions of phosphate or ATP occurred. The rate of exchange of labile phosphate as calculated according to formula (1) gives reasonably consistent figures for the first four measurements but later the value fell.

The amounts of oxygen used, succinate removed, and fumarate and malate formed in this experiment are in the proportion 1 : 1·99 : 1·66. Thus most of the oxygen consumption was due to the conversion of succinate into fumarate and malate. Summaries of three similar experiments on pigeon-breast muscle, rat liver and sheep heart are given in Table 3. These confirm, in general, the findings of the experiment recorded in Table 2. The best experiment, from the point of view of constancy of v (equation 1) is Exp. 1 where pigeon-breast muscle served as the source of the phosphorylation system. Here the value for v was maintained throughout the observation period. In all four experiments the initial phosphorylation quotients are somewhat above 2.

The specific activity of the labile ATP-phosphate approaches in most experiments a value equal to that of inorganic phosphate which indicates that both labile phosphate groups of ATP reacted under the test conditions.

Pigeon-breast muscle preparations oxidizing to fumarate and malate but incapable of oxidizing malate, obtained by washing minced tissue with water or isotonic saline, were found to have lost most of their phosphorylating capacity. ATP is not

Table 3. *Incorporation of* $^{32}PO_4$ *into ATP in tissue suspensions in the presence of succinate*

(Temp., 20°; 4 ml. suspension.)

Exp. 1: pigeon-breast muscle in 45 parts of medium; 0·02 M-succinate; O_2 uptake, 5·33 μl./min. with substrate, 1·83 μl./min. without substrate; average amount of labile phosphate of ATP per cup, 12·0 μmole; of inorganic phosphate, 13·9 μmoles. Succinate removed after 30 min., 412 μl.; fumarate + malate formed after 30 min., 299 μl.; ratio: succinate removed/O_2 used = 2·57.

Time (min.)	2	3	4	6	8	10	15	30
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·175	0·250	0·278	0·410	0·615	0·720	0·940	1·000
v (μmoles/min.)	1·13	1·09	0·93	1·08	1·06	1·24	—	—
Phosphorylation quotient	2·37	2·29	1·95	2·27	2·22	2·60	—	—

Exp. 2: rat-liver suspension in 30 parts of medium; 0·04 M-succinate; O_2 uptake, 8·61 μl./min. with substrate for 30 min., 517 μl./60 min.; 1·87 μl./min. without substrate, 114 μl./60 min.; average amount of labile phosphate of ATP per cup, 18·1 μmoles; of inorganic phosphate, 17·1 μmoles. Succinate removed after 60 min., 895 μl.; fumarate + malate formed, 847 μl.; ratio: succinate removed/O_2 used = 1·73.

Time (min.)	3	6	9	12	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·278	0·452	0·559	0·613	0·723	0·815	0·980
v (μmoles/min.)	1·75	1·51	1·33	1·16	0·88	0·81	
Phosphorylation quotient	2·08	1·80	1·58	1·38	1·05	0·97	

Exp. 3: sheep-heart muscle mince washed twice with 10 vol. 'stock saline' standing 5 min. at 0° each time before filtration through muslin. Before use the mince was ground in a homogenizer and suspended in 15 parts of medium; 0·04 M-succinate; O_2 uptake, 5·95 μl./min. with substrate for 30 min., 360 μl./60 min.; 3·15 μl./min. without substrate, 139 μl./60 min.; average amount of labile phosphate of ATP per cup, 18·6 μmoles; of inorganic phosphate, 20·0 μmoles. Succinate removed after 60 min., 608 μl.; fumarate + malate formed after 60 min., 598 μl.; ratio: succinate removed/O_2 used = 1·69.

Time (min.)	2	3	4	6	8	10	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·143	0·187	0·237	0·242	0·290	0·351	0·460	0·525	0·650
v (μmoles/min.)	1·32	1·16	1·17	0·67	0·78	0·72	0·50	0·39	—
Phosphorylation quotient	2·48	2·18	2·20	1·26	1·47	1·36	0·94	0·74	—

Table 4. *Incorporation of $^{32}PO_4$ into ATP in tissue suspensions in the presence of α-ketoglutarate*

(Temp., 20°; rat-liver suspensions in 30 parts of medium. 4 ml. suspension per cup.)

Exp. 1: 0·02 M-α-ketoglutarate; O_2 uptake with substrate, 4·9 μl./min. during first 45 min., 245 μl./60 min.; without substrate, 175 μl./60 min.; average amount of labile phosphate of ATP per cup, 13·7 μmoles; of inorganic phosphate, 18·8 μmoles. After 60 min. α-ketoglutarate removed, 195 μl.; succinate formed, 50 μl.; fumarate + malate formed, 103 μl.

Time (min.)	2	3	4	6	8	10	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·184	0·271	0·354	0·460	0·543	0·653	0·939	1·000	1·000
v (μmoles/min.)	1·49	1·36	1·26	1·25	1·20	1·26	—	—	—
Phosphorylation quotient	3·41	3·11	2·88	2·86	2·74	2·88	(av. 3·01)		—

Exp. 2: 0·01 M-α-ketoglutarate; O_2 uptake with substrate, 4·28 μl./min. during first 45 min., 242 μl./60 min.; without substrate, 101 μl./60 min.; average amount of labile phosphate of ATP per cup, 13·5 μmoles; of inorganic phosphate, 17·9 μmoles. After 60 min. α-ketoglutarate removed, 189 μl.; succinate formed, ~0; fumarate + malate formed, 155 μl.

Time (min.)	3	6	9	12	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·222	0·418	0·523	0·653	0·854	0·925	1·000
v (μmoles/min.)	1·14	1·18	1·38	1·11	1·30	—	—
Phosphorylation quotient	2·98	3·09	3·61	2·91	3·40	(av. 3·20)	—

Exp. 3: 0·01 M-α-ketoglutarate; O_2 uptake with substrate, 4·80 μl./min. during first 50 min., 281 μl./60 min.; without substrate, 100 μl./60 min.; average amount of labile phosphate of ATP per cup, 16·2 μmoles; of inorganic phosphate, 18·0 μmoles. After 60 min. α-ketoglutarate removed, 170 μl.; succinate formed, 17 μl.; fumarate + malate formed, 119 μl.

Time (min.)	3	6	9	12	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·262	0·425	0·536	0·639	0·810	0·875	1·000
v (μmoles/min.)	1·54	1·38	1·25	1·17	1·29	—	—
Phosphorylation quotient	3·60	3·22	2·92	2·74	3·02	(av. 3·10)	—

maintained in washed tissue suspensions and such preparations are therefore unsuitable for measurements of the phosphorylation quotient.

α-Ketoglutarate as substrate. Three experiments on rat-liver suspension in which α-ketoglutarate was the substrate are shown in Table 4. In general the results resemble those with succinate, but there is no major fall in the values for v with time. The phosphorylation quotients are a little above 3.

A major proportion of the α-ketoglutarate removed was recovered as fumarate and malate and, except in Exp. 2, a smaller proportion as succinate. The data indicate that the greater part of the oxygen uptake was due to the oxidation of α-ketoglutarate to a mixture of fumarate and malate. In Exp. 2 this reaction was responsible for approximately 80 % of the oxidative processes.

Citrate as substrate. Fairly constant values for v were found for the earlier stages of incubation, during which the phosphorylation quotient was in all experiments above 3 (Table 5). Accurate measurement of the substrate changes was not possible because the amounts of citrate removed were small in comparison with the total amount of citrate present. Some fumarate and malate as well as α-ketoglutarate were found but these accounted for not more than 10 % of the oxygen consumption. The main oxidative process in the presence of citrate was therefore probably the complete oxidation of citrate.

Relative reactivity of the two labile phosphate groups

A series of five experiments bearing on the problem of the relative reactivity of the two labile phosphate groups of ATP is given in Table 6. In Exp.1 the specific activities of the terminal and second phosphate groups were determined both separately and together. In the experiment on rat liver both labile phosphate groups exchanged at the same speed. In Exp. 2, pigeon-breast muscle and a commercial ATP preparation (Roche Products Ltd.) were used, a different result being obtained in that the second phosphate group reacted much more slowly than the first. After 10 min. the terminal phosphate was already in equilibrium with inorganic phosphate whilst the specific activity of the second phosphate group was only one-quarter of that of inorganic phosphate.

In Exps. 3–5 only the combined specific activity of both phosphates was determined and in these cases a graph of the ratio specific activity of both labile phosphate groups/specific activity of inorganic phosphate against time gives information on the relative rates of reaction of the two labile phosphate groups. Smooth curves are obtained for Exps. 4 and 5 (as also for Exp. 1) indicating reactivity of both phosphate groups, but the plot of Exp. 3 (and that of Exp. 2) shows a discontinuity at a value of 0·5 for the above ratio. The concentration

Table 5. *Incorporation of $^{32}PO_4$ into ATP in tissue suspensions in the presence of citrate*

(Temp., 20°; rat-liver suspension in 20 parts of medium. 4 ml. suspension per cup.)

Exp. 1: 0·02 M-citrate; O_2 uptake with substrate, 4·64 μl./min., 278 μl. in 60 min.; without substrate, 141 μl. in 60 min.; average amount of labile phosphate of ATP per cup, 16·9 μmoles; of inorganic phosphate, 16·2 μmoles.

Time (min.)	2	3	4	6	8	10	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·193	0·264	0·332	0·430	0·507	0·555	0·770	0·885	1·000
v (μmoles/min.)	1·60	1·49	1·42	1·34	1·24	1·08	0·935	0·975	—
Phosphorylation quotient	3·86	3·60	3·43	3·24	3·00	2·61	2·26	2·36	—

Exp. 2: 0·02 M-citrate and containing 1·0 ml. of 1:1 boiled pigeon-muscle extract; O_2 uptake with substrate, 6·6 μl./min. during first 30 min.; 379 μl. in 60 min.; without substrate, 333 μl. in 60 min; average amount of labile phosphate of ATP per cup, 18·5 μmoles; of inorganic phosphate, 24·4 μmoles; fumarate + malate formed, ~45 μl.

Time (min.)	2	3	4	6	8	10	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·168	0·234	0·312	0·455	0·508	0·571	0·726	0·906	1·000
v (μmoles/min.)	1·97	1·95	1·94	2·02	1·80	1·73	1·75	—	—
Phosphorylation quotient	3·43	3·30	3·29	3·42	3·05	2·93	2·97	—	—

Exp. 3: 0·02 M-citrate; O_2 uptake with substrate, 6·47 μl./min., 388 μl. in 60 min.; without substrate, 195 μl. in 60 min.; average amount of labile phosphate of ATP per cup, 16·5 μmoles; of inorganic phosphate, 17·4 μmoles; α-ketoglutarate + succinate formed, ~48 μl.; fumarate + malate formed, ~50 μl. Citrate removed, ~200 μl.

Time (min.)	2	3	4	6	8	10	20	30	60
Ratio: $\frac{\text{specific activity of labile phosphate}}{\text{specific activity of inorganic phosphate}}$	0·216	0·286	0·405	0·468	0·587	0·613	0·875	0·971	1·000
v (μmoles/min.)	1·97	1·72	1·77	1·48	1·40	1·28	1·22	—	—
Phosphorylation quotient	3·41	2·98	3·06	2·56	2·42	2·22	2·11	—	—

Table 6. *Relative reactivity of the two labile phosphates groups of ATP*

(The total volume of fluid per cup was 4 ml. Unless stated otherwise the ATP preparation was that made according to LePage (1949). Pigeon-breast muscle extract was prepared by keeping freshly minced muscle with 1 part water for 10 min. in a boiling-water bath and filtering at 20°. Other conditions as in previous experiments.)

Exp. 1. Rat-liver suspensions in 30 parts of medium. 0·02 M-α-ketoglutarate.

Time (min.)	3	20	30	60
Ratio: $\frac{\text{specific activity of terminal phosphate of ATP}}{\text{specific activity of inorganic phosphate}}$	0·218	0·815	0·866	1·000
Ratio: $\frac{\text{specific activity of second phosphate of ATP}}{\text{specific activity of inorganic phosphate}}$	0·208	0·806	0·870	1·000
Ratio: $\frac{\text{specific activity of both labile phosphate groups}}{\text{specific activity of inorganic phosphate}}$	0·214	0·805	0·836	1·000

Exp. 2. Pigeon-breast muscle homogenates in 10 parts of medium. 0·04 M-succinate. ATP of Roche Products Ltd.

Time (min.)	5	10	20	40	60
Ratio: $\frac{\text{specific activity of terminal phosphate of ATP}}{\text{specific activity of inorganic phosphate}}$	0·550	1·0	1·0	1·0	1·0
Ratio: $\frac{\text{specific activity of second phosphate of ATP}}{\text{specific activity of inorganic phosphate}}$	0·132	0·244	0·348	0·432	0·540

Exp. 3. Pigeon-breast muscle in 20 parts medium. 0·04 M-succinate. 1 ml. boiled muscle extract.

Time (min.)	5	10	20	60
Ratio: $\frac{\text{specific activity of both labile phosphate groups}}{\text{specific activity of inorganic phosphate}}$	0·610	0·687	0·767	0·906

Exp. 4. Rat-liver suspensions in 20 parts of medium. 0·02 M-citrate. 1 ml. boiled pigeon-muscle extract.

Time (min.)	3	20	30	60
Ratio: $\frac{\text{specific activity of both labile phosphate groups}}{\text{specific activity of inorganic phosphate}}$	0·234	0·726	0·906	1·000

Exp. 5. Pigeon-breast muscle suspension in 45 parts medium. 0·02 M-succinate.

Time (min.)	4	10	15	30
Ratio: $\frac{\text{specific activity of both labile phosphate groups}}{\text{specific activity of inorganic phosphate}}$	0·278	0·720	0·940	1·000

of the suspension was higher than usual in this experiment and the terminal phosphate was in equilibrium with inorganic phosphate in less than 5 min., whilst the second phosphate was not in equilibrium even after 60 min.

These observations lead to the following conclusion: in both liver (Exps. 1 and 4) and pigeon-muscle (Exp. 5) suspensions two phosphate groups of ATP can react at equal rates, but under certain conditions the rate of reaction of the second phosphate may be slower than that of the first in the case of pigeon-breast muscle, e.g. when either boiled muscle extract (Exp. 3) or the ATP preparation of Roche Products Ltd., instead of the preparation according to LePage (Exp. 2), is present. Boiled muscle extract or Roche Products' ATP have no such effect on the reactivity of the second phosphate in liver suspensions.

The statement made by Krebs, Johnson, Eggleston & Hems (1951) that only one phosphate of ATP reacts in muscle but two in liver is thus correct only under special conditions. The statement was based on experiments in which the ATP preparation of Roche Products Ltd. was used and is also correct when boiled muscle extract is present.

Boiled muscle extract was examined when it was found that the reactivity of the second phosphate in muscle suspension depended on the ATP preparation. It is evident from this finding that the slow reactivity of the second phosphate is due to an impurity accompanying ATP, and the fact that muscle extract produces the same effect indicates that it is an impurity derived from the tissue and not from the reagents introduced in the isolation of ATP. The nature of the impurity is so far obscure. The boiled extract contains an active myokinase which might be expected to increase the reactivity of the second phosphate rather than to reduce it.

Anaerobic phosphorylation

The quantitative significance of the phosphorylation quotients obtained in the preceding part of this paper depends on the absence of anaerobic phosphorylation under the test conditions. Whether this occurred was tested by repeating anaerobically some of the experiments recorded in Tables 3 and 4. There was a rapid decrease in the concentration of ATP in all anaerobic experiments (conditions as Exp. 2, Table 3, and Exp. 2, Table 4). In rat-liver suspension 35 % of the original labile ATP phosphate was lost in 5 min. at 20°. The remaining ATP contained small but measurable quantities of ^{32}P, but the total count after 5 min. was only 9 % of the aerobic value in the presence of succinate and 13 % in the presence of α-ketoglutarate. As no steady state existed, the turnover rate could not be calculated, but the conclusion may be drawn that such anaerobic phosphorylation as did occur was too slow to vitiate appreciably the calculations of the phosphorylation quotients.

DISCUSSION

Applicability of theory. A constancy of the v values calculated from different pairs of t and x may be regarded as support, though not as proof, of the theory. Satisfactory constancy throughout the major part of the incubation period was obtained in experiments where α-ketoglutarate was the substrate (Table 4) and also in Exp. 1 of Table 3 where succinate was the substrate and pigeon-breast muscle the source of enzyme. Thus the theory is satisfactorily obeyed under some conditions. Where deviations are observed, such as the fall of v occurring in the later period of incubation (e.g. in some of the experiments with succinate or citrate), this is probably due to complicating factors not operating in the early part of incubation, or in the presence of α-ketoglutarate. The fact that the phosphorylation quotients obtained are in essential agreement with the highest values found by other authors using entirely different methods gives further support to the theory.

Phosphorylation quotient for individual stages. In none of the experiments was the oxidative process strictly limited to a single stage, but when succinate was the substrate the major part of the oxidative reactions was the conversion of succinate into fumarate; not less than 80 % of the oxygen uptake accounted for this reaction. In all experiments with succinate as substrate the phosphorylation quotient was near 2, generally a little above this value; a quotient of 2 for the reaction succinate → fumarate may therefore be taken as the best available value. Measurements with different methods by other authors (Cross, Taggart, Covo & Green, 1949; Eiler & McEwan, 1949; Hunter & Hixon, 1949*a*, *b*; Copenhaver & Lardy, 1952) have generally yielded somewhat lower phosphorylation quotients, the highest previously recorded values being 1·7–1·8.

Phosphorylation quotients of the order of 3 were obtained when α-ketoglutarate was the substrate and when the main reaction was the conversion of α-ketoglutarate into a mixture of fumarate and malate (see Table 4). This reaction involves two stages and if the phosphorylation quotient is 2 for oxidation of succinate to fumarate and malate, and 3 for the conversion of α-ketoglutarate into fumarate and malate, it must be 4 for the first step of the oxidation of α-ketoglutarate, i.e. its conversion into succinate. This conclusion is in accordance with those of previous workers (Cross *et al.* 1949; Hunter & Hixon, 1949*a*, *b*; Copenhaver & Lardy, 1952; especially Hunter, 1951).

The observations with citrate as substrate (Table 5) do not supply direct information on the phosphorylation quotient of individual stages as most of the citrate appeared to undergo complete oxidation. The figure of 3 observed supports other evidence showing that up to three phosphate molecules can react, on an average, per atom of oxygen consumed during the complete oxidation of intermediate metabolites (see authors quoted above and Judah, 1951; Lehninger & Smith, 1949; Johnson & Lardy, 1950).

Thermodynamic efficiency of oxidative phosphorylation. Estimates of the free-energy changes for the individual oxidative steps and for the hydrolysis of ATP holding for conditions similar to those prevailing in the experiments (pH 7·0–7·4; 0·2 atm. O_2; 0·5 atm. CO_2; 0·005 M-inorganic phosphate; ratio ATP/ADP = 10) are given by Burton & Krebs (1953). In the experiments reported in this paper the concentration of the organic substrates changes during the incubation, and, as any corrections taking into consideration substrate concentration are relatively small, no adjustments are made in the following calculation. According to Burton & Krebs (1953) ΔG of the conversion of α-ketoglutarate into succinate is 69·8 kcal. at equal concentrations of α-ketoglutarate and succinate, and for the synthesis of ATP 14 kcal. Assuming a phosphorylation quotient of 4 the efficiency is therefore

$$4 \times 14 \times 100/69{\cdot}8 = 80\,\%.$$

Assuming a phosphorylation quotient of 2 for the conversion of succinate into fumarate the efficiency is $2 \times 14 \times 100/35{\cdot}7 = 78\,\%$. In these calculations it is also assumed that the free energy of hydrolysis of ATP is not utilized for the synthesis of ATP under the test conditions, an assumption supported by the observation that the rate of anaerobic incorporation of $^{32}PO_4$ into ATP is very slow.

High efficiencies can be expected only if most of the respiration serves to resynthesize pyrophosphate bonds, which would imply that the rate of respiration is controlled by the rate at which the substrates for the synthesis of ATP become available. There is in fact evidence supporting this conception (Recknagel & Potter, 1951; Kaplan, 1951; Lardy & Wellman, 1952).

Phosphorylation coupled with the oxidation of cytochrome c. When succinate is oxidized by molecular O_2 about one-third of the total free-energy change occurs in the reactions between cytochrome *c* and succinate and two-thirds in the reactions between cytochrome *c* and oxygen (Ball, 1944; Hunter, 1951). The free energy liberated between the stages lying in the interaction of cytochrome *c* and succinate can thus at most account for the formation of one pyrophosphate bond. If two are formed, the free energy of the oxidation of cytochrome must also be utilized for the synthesis of pyrophosphate bonds, as already emphasized by Hunter (1951). An analogous calculation for the case of α-ketoglutarate also suggests, although not as forcibly, that the free energy of the oxidation of cytochrome *c* by oxygen is utilized.

Reactivity of the two phosphate groups of ATP. The data in Table 6 show that two phosphate groups of ATP are, under some conditions, equally labile, as judged by the rate of interchange with inorganic phosphate, whilst under others the terminal group reacts more rapidly than the second. Animal tissues are known to be able to phosphorylate ADP according to the reaction

$$\mathrm{ADP} + \mathrm{P} \rightarrow \mathrm{ATP}, \qquad (4)$$

and to possess the enzyme myokinase (Kalckar, 1943, 1951; Kotelnikova, 1948; Colowick, 1951), which converts ADP reversibly into AMP and ATP,

$$\mathrm{AMP} + \mathrm{ATP} \rightleftharpoons 2\mathrm{ADP}. \qquad (5)$$

The balance of these two reactions (reaction 4 occurring twice) is the synthesis of ATP from AMP and 2P. These two known reactions, together with action of ATP-ases, could account for the observations on the exchange of two labile phosphate groups reported in this paper (see also Novikoff, Hecht, Podber & Ryan, 1952; Barkulis & Lehninger, 1951*b*). Under conditions when both phosphate groups exchange at equal rates the myokinase reaction must be rapid in comparison to the synthesis of ATP by reaction (4). A low reactivity of the second phosphate group can be accounted for by the inhibition, or absence, of myokinase.

Whilst there is so far no evidence of a phosphorylation of AMP by a mechanism other than reaction (5), the possibility of a more direct reaction between AMP and inorganic phosphate cannot be ruled out.

SUMMARY

1. The rate of incorporation of (^{32}P) potassium dihydrogen phosphate ($KH_2{}^{32}PO_4$) into adenosinetriphosphate (ATP) in respiring tissue suspensions was measured under conditions where a steady state existed with respect to the concentrations of inorganic phosphate and ATP. From these measurements the rate at which the pyrophosphate bonds of ATP are formed was calculated. The theoretical and practical aspects of the procedure are discussed.

2. From the rate of formation of pyrophosphate bonds and of the simultaneous oxygen uptake the ratio equivalents of organic phosphate formed/atoms of oxygen consumed, the 'phosphorylation quotient', was calculated. Its value was found to be near 2 when the main oxidative process was the oxidation by oxygen of succinate to fumarate and malate and near 3 when the main oxidative process

was the oxidation of α-ketoglutarate to fumarate and malate. The value of the ratio for the oxidation of ketoglutarate to succinate must therefore be 4. These results confirm the highest values obtained by previous authors with entirely different procedures.

3. The thermodynamic efficiency of the oxidative phosphorylation was calculated to be of the order of 80 % when succinate or ketoglutarate was the main substrate of oxidation. Efficiencies of this order are possible only if the free energy of the reactions between oxygen and the cytochromes is utilized.

4. In liver suspensions two phosphate groups of ATP reacted with almost identical speeds. In pigeon-breast muscle suspensions the reactivity of the second phosphate group was under some conditions much slower than that of the terminal group. The slow reactivity of the second phosphate group in muscle tissue appears to be due to the presence of a specific inhibitor of one of the enzymes concerned.

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APPENDIX

Calculation of Reaction Rates in Steady Systems

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Elaboration of problem 1. This problem represents the simplest case and its nature is fully stated in the preceding paper to which reference should be made. In the previous treatment v is calculated from the initial radioactivity (x_0) of a and measurements of the radioactivity x of a at the time t. Alternatively v can be calculated from determinations of the radioactivity y of b. This may serve as a check in some cases. In others it may be a matter of convenience when the isolation and determination of the radioactivity of a offers greater difficulties than the determination of the radioactivity of b.

Three cases may be distinguished.

(1) One measurement of y is used covering the time between t_0 and t:

$$v=\frac{ab}{t(a+b)}\ln\frac{bx_0}{bx_0-y(a+b)}.$$

(2) Two measurements of y are used (y_1, y_2, at t_1, t_2). In this case the changes of y occurring between t_1 and t_2 are relevant. This may be advantageous if, owing to a lag period, the starting point of the reaction is not clearly defined or if there is a time lag in the establishment of the steady state:

$$v=\frac{ab}{(t_2-t_1)(a+b)}\ln\frac{bx_0-y_1(a+b)}{bx_0-y_2(a+b)}.$$

(3) No value for x_0 is required but three measurements of y are used at t_1, t_2, t_3, the time intervals between t_1, t_2 and t_3 being equal:

$$v=\frac{ab}{m(a+b)}\ln\frac{y_2-y_1}{y_3-y_2},$$

where $m=t_2-t_1=t_3-t_2$. For accurate determinations of v, y should not be near its steady state value.

Problem 2.

$$M+2N=MNN.$$

This involves (at least) two separate stages the nature of which may not be known for certain, but three different possibilities are discussed:

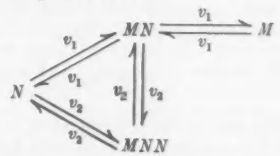
(1) (*a*) $M+N\rightleftharpoons MN$,
(*b*) $MN+N\rightleftharpoons MNN$.

(2) (*a*) $2[MN+N\rightleftharpoons MNN]$,
(*b*) $M+MNN\rightleftharpoons 2MN$.

(3) (*a*) $2[M+N\rightleftharpoons MN]$
(*b*) $2MN\rightleftharpoons M+MNN$.

The most likely biological mechanisms in the case of the synthesis of ATP is (2) but (1) cannot be excluded. There is no evidence in support of (3). Only cases (1) and (2) are therefore considered.

Case 1. The reactions taking place in this case are diagrammatically as follows:



It is assumed that a steady state exists, so that the rates of formation and disappearance of each substance are equal.

Let v_1 = rate at which N exchanges with MN;
v_2 = rate at which N exchanges with MNN;
a = amount of, x = radioactivity of, N;
b = amount of, y = radioactivity of, MNN;
c = amount of, z = radioactivity of, MN.

It is assumed that c is small in comparison to a and b and that v_1v_2 is large in comparison to c/t. Required is v_1+v_2.

It follows from the above assumptions that

$$\frac{dx}{dt}=-\frac{(v_1+v_2)x}{a}+\frac{v_2y}{b}+\frac{v_1z}{c}, \tag{1}$$

$$\frac{dy}{dt}=-\frac{2v_2y}{b}+\frac{v_2x}{a}+\frac{v_2z}{c}, \tag{2}$$

$$\frac{dz}{dt}=-\frac{(v_1+v_2)z}{c}+\frac{v_1x}{a}+\frac{v_2y}{b}, \tag{3}$$

$$x+y+z=x_0. \tag{4}$$

Substitution of (4) in (1) and (3) gives:

$$\left(\mathrm{D}+\frac{v_1+v_2}{a}+\frac{v_2}{b}\right)x+\left(\frac{v_2}{b}-\frac{v_1}{c}\right)z=\frac{v_2x_0}{b}\quad\left(\mathrm{D}=\frac{d}{dt}\right), \tag{5}$$

$$\left(\frac{v_2}{b}-\frac{v_1}{a}\right)x+\left(\mathrm{D}+\frac{v_1+v_2}{c}+\frac{v_2}{b}\right)z=\frac{v_2x_0}{b}. \tag{6}$$

Elimination of z gives

$$\left\{\mathrm{D}^2+\mathrm{D}\left(\frac{v_1+v_2}{a}+\frac{2v_2}{b}+\frac{v_1+v_2}{c}\right)+v_2(2v_1+v_2)\left(\frac{1}{bc}+\frac{1}{ca}+\frac{1}{ab}\right)\right\}x=\frac{v_2(2v_1+v_2)}{bc}x_0. \tag{7}$$

The initial conditions for x are:

$$x=x_0,\quad \mathrm{D}x=-\frac{(v_1+v_2)}{a}x_0.$$

The exact solution is therefore of the form

$$x=\frac{ax_0}{a+b+c}+Ae^{-\alpha t}+Be^{-\beta t},$$

where $\dfrac{(b+c)x_0}{a+b+c}=A+B,\quad \dfrac{(v_1+v_2)x_0}{a}=\alpha A+\beta B,$

and α, β are the roots of the quadratic equation

$$\theta^2-\theta\left(\frac{v_1+v_2}{a}+\frac{2v_2}{b}+\frac{v_1+v_2}{c}\right)+v_2(2v_1+v_2)\left(\frac{1}{bc}+\frac{1}{ca}+\frac{1}{ab}\right)=0. \tag{8}$$

The result is too complicated for a simple adaptation to experimental data, but if c is small in comparison to a and b, a suitable approximation to x and z may be obtained.

One root α is of the form $k_1+o(c)$ and the other root β is of the form $l_1/c+l_2+o(c)$, where k_1 and l_1 are both positive.

Substitution of these forms in the equations for A and B shows that A is of the form $A_0+o(c)$ and B is of the form $o(c)$.

The value of x is therefore of the form

$$\frac{ax_0}{a+b}+o(c)+[A_0+o(c)]\,e^{[-k_1+o(c)]t}+o(c)\exp\left\{-\left[\frac{l_1}{c}+l_2+o(c)\right]t\right\}.$$

The leading term in the approximation is obtained by letting c tend to $+0$ and this then takes the form

$$x=\frac{ax_0}{a+b}+A_0e^{-k_1t}.$$

Finally equation (5) shows that z/c has a form similar to x when c tends to $+0$.

x is found from equation (7) by equating the coefficient of $1/c$ to zero. This gives

$$\left\{(v_1+v_2)\,\mathrm{D}+v_2(2v_1+v_2)\left(\frac{1}{a}+\frac{1}{b}\right)\right\}x=\frac{v_2(2v_1+v_2)\,x_0}{b},$$

the solution of which is:

$$x=\frac{ax_0}{a+b}+\frac{bx_0}{a+b}\exp\left[-K\left(\frac{1}{a}+\frac{1}{c}\right)t\right], \tag{9}$$

where $$K=\frac{v_2(2v_1+v_2)}{v_1+v_2}.$$

Substitution of x from (9) into (5) will give

$$z=\frac{cx_0}{a+b}-\frac{cx_0}{a+b}\left(1-\frac{a+b}{a}L\right)\exp\left[-K\left(\frac{1}{a}+\frac{1}{b}\right)t\right], \tag{10}$$

where $$L=\frac{v_1}{v_1+v_2}.$$

It is assumed in (9) that terms c/a, c/b are negligible and in (10) that c^2/a^2, c^2/b^2 are negligible.

Also it is assumed in (10) that the term $e^{-l_1(t/c)}$, is negligible. Since $l_1=v_1+v_2$, the neglect of this term implies that $(v_1+v_2)\,t$ is large in comparison to c. This means that a sufficient time has elapsed from $t=0$ to ensure that the flux into and from c is large compared to c.

Rearranging (9) gives:

$$K=\frac{ab}{t(a+b)}\ln\frac{bx_0}{x(a+b)-ax_0}.$$

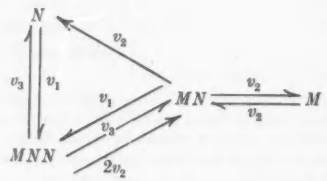
The main result is that (9) is formally identical with the formula derived for the single step case, but the significance of the constant is somewhat different. Thus if a plot of $\ln\dfrac{bx_0}{x(a+b)-ax_0}$ against $\dfrac{t(a+b)}{ab}$ gives a straight line the underlying mechanism may be either a one-step or a two-step reaction.

It should be noted that the K is equal to

$$v_1+v_2-v_1^2/(v_1+v_2)$$

and is therefore a minimum value for the effective speed.

Case 2. The reactions taking place are diagrammatically shown in scheme 2.



Required are v_2+v_3 (which equal v_1). The assumptions are the same as in case 1. The equations are:

$$\frac{dx}{dt}=v_2\frac{z}{c}-v_1\frac{x}{a}+v_3\frac{y}{b}, \tag{1}$$

$$\frac{dz}{dt}=-v_2\frac{z}{c}-v_1\frac{z}{c}+(v_3+2v_2)\frac{y}{b}, \tag{2}$$

$$x+y+z=x_0. \tag{3}$$

The method used for case 1 is applicable here, since the equations are similar in form.

Substitution from (3) into (1) and (2) gives:

$$\left(\mathrm{D}+\frac{v_2+v_3}{a}+\frac{v_3}{b}\right)x+\left(\frac{v_3}{b}-\frac{v_2}{c}\right)z=\frac{v_3x_0}{b}, \tag{4}$$

$$\frac{v_3+2v_2}{b}x+\left(\mathrm{D}+\frac{v_3+2v_2}{b}+\frac{v_3+2v_2}{c}\right)z=\frac{v_3+2v_2}{b}x_0. \tag{5}$$

Taking $z+0(c)$

$$\left(\mathrm{D}+\frac{v_2+v_3}{a}+\frac{v_3}{b}\right)x-\frac{v_2z}{c}=\frac{v_3}{b}x_0, \tag{6}$$

$$\frac{x}{b}+\frac{z}{c}=\frac{x_0}{b}, \tag{7}$$

i.e. $$\left(\mathrm{D}+\frac{v_2+v_3}{a}+\frac{v_2+v_3}{b}\right)x=\frac{v_2+v_3}{b}x_0.$$

The solution

$$x=\frac{ax_0}{a+b}+\frac{bx_0}{a+b}\exp\left[-(v_2+v_3)\left(\frac{1}{a}+\frac{1}{b}\right)t\right]. \tag{8}$$

Substitution in (7) gives:

$$z=\frac{cx_0}{a+b}-\frac{cx_0}{a+b}\exp\left[-(v_2+v_3)\left(\frac{1}{a}+\frac{1}{b}\right)t\right]. \tag{9}$$

Rearranging (8) gives:

$$v_2+v_3=v_1=\frac{ab}{t(a+b)}\ln\frac{bx_0}{x(a+b)-ax_0}.$$

This formula is again formally identical with that derived for the single-step mechanism. In the present case the constant signifies the actual rate at which the labile phosphate exchanges.

Studies on the Proteins of Fish Skeletal Muscle

1. ELECTROPHORETIC ANALYSIS OF CODLING EXTRACTS OF LOW IONIC STRENGTH

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Relatively little is known of the characteristics of fish muscle proteins, though a few preliminary studies have been made. Roth (1947) has demonstrated that the 'classical' muscle protein fractions of some freshwater fish resemble in their general properties the corresponding fractions of rabbit or frog muscle. Using the methods of the Liége Laboratory, Hamoir (1949, 1951*b*) has examined several types of carp muscle extract by electrophoresis and ultracentrifugation, and has compared the results with those from rabbit muscle. Hamoir (1951*a*, *c*) has also described one of the few well-characterized fish muscle proteins, nucleotropomyosin; this appears to be a nucleic acid derivative of Bailey's (1948) tropomyosin.

Reay (1935), Reay & Kuchel (1936), and more recently Subba Rao (1948), and Dyer, French & Snow (1950) have quantitatively analysed skeletal muscle of several marine species of fish by methods based on Bate-Smith's (1937) scheme.

Detailed electrophoretic studies of extracts of low ionic strength, such as are described in this paper, have been carried out on rabbit muscle by Jacob (1947, 1948) and Bosch (1951).

EXPERIMENTAL

Electrophoresis was conducted in the Tiselius apparatus at +0·5°. The Philpot-Svensson diagonal edge optical system was used throughout. All the results were obtained using the tall-form cell, and the flow-through modification of Kekwick, Lyttleton, Brewer & Dreblow (1951) has been used to facilitate repetition of runs.

Codling (*Gadus callarias* Linn.) were used exclusively. The fish (30–50 cm. in length) were caught from November to May a few miles off Aberdeen, and rested in a shore tank. They were killed by decapitation, and then skinned and filleted in the commercial fashion. The fillet represents the whole of the musculature on both sides of the fish. Occasionally, fish were anaesthetized by urethane before decapitation in order to prevent struggling, but the electrophoretic patterns obtained from such fish were not noticeably different from those of non-anaesthetized fish, nor were the patterns obtained from fish which had been exsanguinated via the tail. The fillets were immediately transferred to a cold room (0°) and finely minced. All subsequent operations were carried out at 0°.

The mince was stirred for 1–3 hr. in phosphate buffer (1–3 ml./g. mince, depending upon ionic strength), and centrifuged at 4000 *g* for 30 min. Generally, one-half to two-thirds volume of clear supernatant was obtained in this way. The extract was then dialysed in cellophan tubes for about 48 hr. with frequent changes of outer liquid. Normally the extracts ($I \leqslant 0{\cdot}2$) remained quite clear, and only small amounts of protein precipitated on dialysis. Before electrophoresis the extract was always centrifuged at 4000 *g* for 30 min., and, if necessary, the N content was adjusted by dilution to below 4 mg./ml. The conductivity of the outer dialysis liquid, which was used as supernatant in the electrophoresis cell, was measured at 0°. The values of the extract and supernatant were measured with the glass electrode at 10°; recorded pH values are the mean of these values.

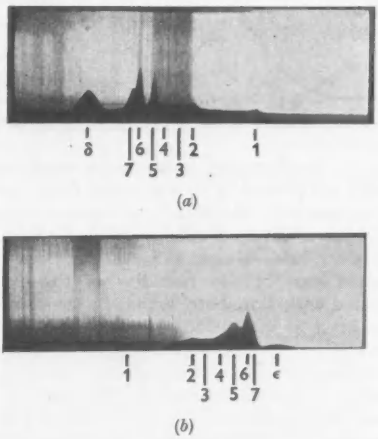


Fig. 1. Electrophoretic diagram of an extract of codling skeletal muscle at 0·05*I*, pH 7·46. (*a*) ascending limb; (*b*) descending limb. Exp. 43/52: field strength, 7·35 V./cm.; duration of electrophoresis, 4140 sec.

RESULTS

Extracts were made at 0·05, 0·1 and 0·2*I* in order to find the best conditions for electrophoresis, and to observe any changes in protein composition with changes in ionic strength. These extracts contain the globulin X, myogen and myoalbumin fractions of muscle. The pH was kept close to 7·5 throughout.

Extracts at 0·05*I* (Fig. 1). Extractant and diffusate fluid: 0·0156 M-Na_2HPO_4 + 0·0035 M-KH_2PO_4, pH 7·5 (1 ml./g. mince).

The more obvious components have been numbered arbitrarily from 1 to 7 in decreasing order of mobility. There was no simple correlation between the diagrams of codling and rabbit, therefore neither the nomenclature of Jacob (1947) nor that of Bosch (1951) has been used. It is possible to divide the codling diagram into the same general mobility groups of I–III as for the rabbit and frog diagrams (Jacob, 1945, 1947). Group III, with the highest mobility, separates very quickly from the initial boundary, and comprises in the present case only one small and usually diffuse peak, 1. Group II, of average mobility, subdivides into three peaks, 2, 3 and 4. Between groups III and II is situated an ill-defined region of refracting material, the top of which lies almost parallel to the base-line. Following Jacob (1947) this region was designated *sp* (space), contributing up to about 7 % of the total area of the diagram. In most cases, there was a suggestion of a small component in *sp* somewhat faster than 2, but it was too ill-defined to warrant separate designation. Group I subdivides into peaks 5–7, and contributes about 65 % of the total area. A small part of the turbidity of the extracts migrated with 1 in both limbs; in the ascending limb, most of the turbidity migrated with 2, the apex of 2 sharply marking the edge of the turbidity; in the descending limb, the turbidity migrated with 2 and 3, increasing from 2 to 3. Especially on prolonged electrophoresis, further separation of peaks between 5 and 6 occasionally occurred, and the gradual tailing off of the slowest part of the diagram indicated the presence of small components slower than 7. That the division into these seven components did not fully represent the complexity of the diagram is illustrated by Fig. 2 in which the ascending side of Exp. 43/52 has been analysed by constructing approximate error curves (Svedberg & Pedersen, 1940; Wiedemann, 1947). There is a large degree of arbitrariness in the selection of appropriate error curves for the complex diagrams met with here, and the disposition and number of the overlapping curves varies from one diagram to another. The simpler perpendicular extrapolation method (Tiselius & Kabat, 1939) has therefore been used for estimating the proportions of the components. The contributions of the components to the total area of the diagram for several analyses are shown in Table 1.

All the components in both limbs moved anodically at pH 7·5, consequently there was no difficulty in accounting for the stationary δ or ϵ boundaries. The results of the present experiments bear no relation to the protein concentration, to small variations in pH or to the duration of electrophoresis. The variations between individual values of the amount of the same component were fairly large and probably outside the experimental error, but were least for experiments with the same extract or with the same fish, which indicates that the variations may be physiological in nature. Variations in the electrophoretic composition of rabbit muscle extracts have been explained on the same basis (Jacob, 1947; Bosch, 1951).

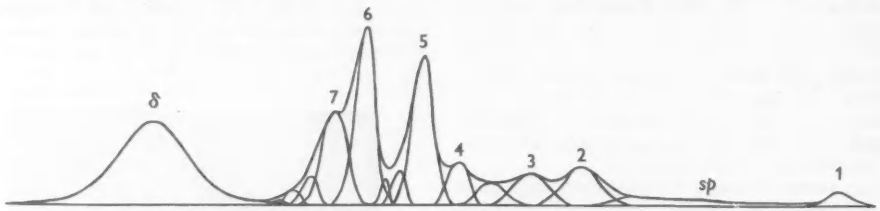


Fig. 2. Tracing of ascending limb diagram of Exp. 43/52 showing construction of approximate error curves.

The pronounced variations in the amount of 2 may be due to another factor which becomes evident when aged or partially heat-denatured extracts are examined. Extracts which have been kept at 0° for longer periods than about 7 days, or at room temperature for about 18 hr. became more opaque, but very little more protein could be removed from them by centrifugation. The diagrams of such modified extracts appear normal except for a pronounced increase in the proportion of 2, and small and variable decreases in the proportions of all the other components (Table 2). Most of the turbidity of aged extracts migrated precisely with 2. Jacob (1948) also noted an increase in the turbidity of group II, whilst Bosch (1951) found that the only change brought about by ageing was a decrease in the slowest component of group I. It is possible that denaturation in phosphate buffers is associated with the formation of a complex whose mobility coincides with that of 2; the large variations of 2 in 'normal' extracts might then be accounted for by incipient denaturation.

Extracts made at ionic strengths sufficient to bring the actin-myosin group into solution (0·3–0·5 I) and then dialysed to 0·05 I (Exp. 36/52) gave diagrams similar to those of extracts at 0·05 I, though the low protein content made precise analysis difficult.

Table 1. *Composition of codling extracts, 0·05 I*

(A, ascending diagram; D, descending diagram.)

Exp. no.	pH	N content (mg./ml.)		Component, % total area							
				1	*sp*	2	3	4	5	6	7
23/52*	7·49	3·58	A	2·9	7·3	8·3	6·6	8·6	24·2	30·8	11·3
			D	2·1	4·8	6·0	6·9	12·3	26·2	30·4	11·3
24/52*	7·49	3·58	A	—	—	7·1	6·8	7·8	25·9	32·7	11·7
			D	4·0	3·7	7·9	7·2	11·4	25·5	29·4	10·9
36/52†	7·54	0·89	D	5·9	6·0	11·4	12·0		22·2	42·5	
41/52	7·40	4·10	A	—	7·0	7·6	5·2	8·4	25·7	32·8	10·3
			D	3·4	4·3	7·9	6·0	8·9	27·4	31·5	10·6
43/52	7·46	3·30	A	3·5	6·6	10·4	7·6	7·9	22·4	26·5	15·1
			D	4·5	7·0	12·5	7·0	10·7	24·9	22·2	11·2
57/52‡	7·55	3·10	A	2·5	6·6	4·7	6·8	9·6	26·0	31·2	12·6
58/52	7·58	3·31	A	2·6	5·4	5·0	6·6	9·1	26·1	33·6	11·6
			D	3·2	6·1	7·9	8·7	10·3	25·7	27·8	10·3
59/52‡	7·59	3·61	A	2·4	7·2	6·3	7·0	8·3	27·7	30·9	10·2
			D	3·0	5·2	5·4	7·2	10·8	25·8	31·3	11·2
60/52	7·59	3·04	D	4·1	7·1	6·1	6·6	8·7	24·9	31·6	10·9
Arith. mean				3·5	5·9	7·6	6·9	9·5	25·4	30·2	11·4
S.D.§				1·0	1·2	2·3	0·8	1·4	1·3	2·9	1·3

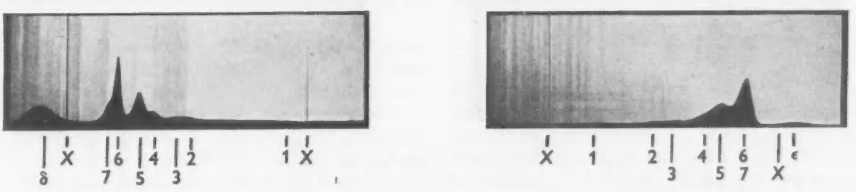
* Same extract. † Obtained by dialysis of an extract at 0·3 *I*.
‡ Same fish. § Standard deviation $= \sqrt{[\Sigma d^2/(n-1)]}$.

Table 2. *Composition of partially denatured extracts 0·05 I*

(A, ascending diagram; D, descending diagram.)

Exp. no.	pH	N content (mg./ml.)		Component, % total area							
				1	*sp*	2	3	4	5	6	7
42/52*	7·43	3·90	A	0·9	7·4	13·8	5·9	6·8	25·0	29·8	10·4
			D	1·6	5·3	13·4	6·2	10·9	25·6	26·9	10·3
44/52†	7·42	3·60	A	2·4	7·7	17·5	8·6		21·1	42·7	

* 7 days at 0°. † 18 hr. at room temperature.



(*a*) (*b*)

Fig. 3. Electrophoretic diagram of an extract at 0·1 *I*, pH 7·45. (*a*) ascending limb; (*b*) descending limb. Exp. 2/52: field strength, 4·47 V./cm.; duration of electrophoresis, 12060 sec. The lines *X* denote the position on the cell faces of engravings which are used as markers.

Extracts at 0·1 *I* (Fig. 3). Extractant and diffusate fluid: 0·0312 M-Na_2HPO_4 + 0·007 M-KH_2PO_4, pH 7·5 (1 ml./g. mince).

The diagrams as a whole were less clearly defined than those at 0·05 *I*. Group III was very diffuse, and in most cases could only be estimated immediately after separation from the initial boundary. In group II, 3 did not separate clearly and appeared to be partly associated with both 2 and 4, whilst in group I, 7 did not separate at all on the descending side.

Evaluation of the diagrams (Table 3) shows that the leading components (1–4) contribute less and the slower components (5–7) more to the total area than

Table 3. *Composition of codling extracts, 0·1 I*

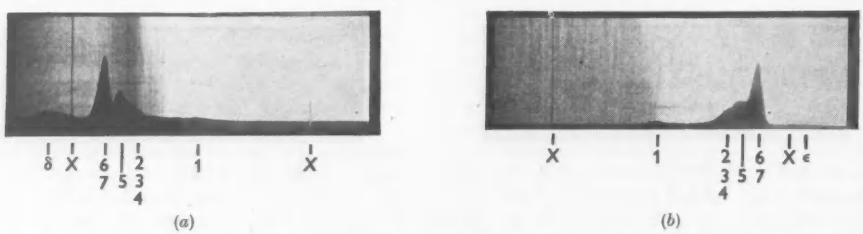
(A, ascending diagram; D, descending diagram.)

Exp. no.	pH	N content (mg./ml.)		Component, % total area 1	*sp*	2	3	4	5	6	7
3/51	7·5	—	A	—	—	3·7	4·3	7·8	27·9	35·6	12·0
			D	3·4	5·3	5·3	9·8		29·1	47·1	
4/51	7·5	—	A	—	—	8·4		8·4	28·4	36·1	12·7
			D	—	2·9	4·8		10·5	27·0	51·8	
6/51	7·5	—	A	—	—	7·4		9·7	26·5	37·8	9·7
			D	—	5·9	5·3		6·4	27·4	52·0	
2/52	7·45	5·10	A	—	—	2·9	4·3	7·4	27·0	39·4	10·4
			D	2·4	6·1	6·1		8·7	26·9	49·8	
Arith. mean				2·9	5·0	15·2			27·5	37·2	11·2

in the case of 0·05 *I* extracts. This is probably a consequence of the boundary anomalies rather than an actual change in composition of the extract, since extracts at 0·1 *I* when dialysed to 0·05 *I* lose little protein and have similar composition to 0·05 *I* extracts. The moving boundary theories of Svensson (1946) and Dole (1945) predict that the amounts of leading and slowest ions, as judged from their refractive index increments, will be diminished and enhanced respectively on passing from lower to higher ionic strength at equivalent protein concentration. The same tendency is shown at 0·2 *I* though the interpretation of the diagrams at this ionic strength is somewhat ambiguous.

Extracts at 0·2 *I* (Fig. 4). Extractant and diffusate fluid: 0·1 M - KCl + 0·0312 M - Na_2HPO_4 + 0·007 M - KH_2PO_4, pH 7·5 (2 ml./g. mince).

An extract at 0·05 *I* dialysed to 0·2 *I* (Exp. 49/52, Table 4) gave diagrams which were very similar in appearance to those obtained with 0·2 *I* extracts and therefore it was concluded that the gross differences between extracts at 0·2 *I* and at 0·05 and 0·1 *I* were due to the influence of increased ionic strength on electrophoretic separation rather than to differences in the protein composition of the extracts. However, solutions at 0·2 *I* do extract more protein than solutions at 0·05 *I*, and on dialysis of an 0·2 *I* extract to 0·05 *I* about one-quarter of the protein is precipitated. It has not been possible to apportion the extra protein extracted at 0·2 *I* among the components with great exactitude. Both the proportions of group II and of 6 and 7 appear to increase in relation to the total at 0·2 *I*. It is possible that group II may represent in



(*a*) (*b*)

Fig. 4. Electrophoretic diagram of an extract at 0·2 *I*, pH 7·41. (*a*) ascending limb; (*b*) descending limb. Exp. 4/52: field strength, 3·94 V./cm.; duration of electrophoresis, 13320 sec.

The diagrams showed much less detail than those at 0·05 or 0·1 *I*, even after prolonged electrophoresis. Group III was again diffuse and group II consisted of only one distinct peak, with which the turbidity of the extract moved precisely. In group I, 5 and 6 could be distinguished but 7 separated on only one occasion. The evaluation of these extracts is shown in Table 4.

part the myosin group of proteins since an electrophoretic component with the properties of myosin, and which travels at the rate of group II, becomes evident at 0·3 *I* (Connell, unpublished). Even at 0·2 *I* a small amount of protein is dissolved from a washed actomyosin gel by phosphate buffer and, although difficult to observe in the electrophoresis apparatus, it travels with a mean mobility between

Table 4. *Composition of codling extracts, 0·2 I*

(A, ascending diagram; D, descending diagram.)

Exp. no.	pH	N content (mg./ml.)		Component, % total area 1	*sp*	2?	3?	4?	5	6	7
4/52*	7·41	4·20	A	3·5	6·4		8·6		27·3	54·2	
			D	3·1		18·6			21·8	56·5	
33/52	7·44	2·20	A	5·1		16·0			23·5	55·4	
			D	5·1		17·0			23·2	54·7	
47/52	7·32	2·30	A	5·0	4·0		9·9		27·1	54·0	
			D	5·0	5·1		11·6		28·4	49·9	
49/52†	7·44	3·30	A	4·8	6·0		7·2		28·5	53·5	
Arith. mean‡				4·6		16·2			25·2	54·1	

* 1 ml. extractant per g. muscle. † Extract at 0·05 *I* dialysed to 0·2 *I*. ‡ Excluding Exp. 49/52.

Table 5. *Correction of ascending mobilities*

(A, ascending mobility; C, corrected ascending mobility; D, descending mobility; Δ_a and Δ_d are the total traced areas on the ascending and descending sides, respectively.)

	Δ_a	Δ_d	δ	ϵ		Mobility (10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$) 1	2	3	4	5	6	7
Exp. no.	Arbitrary units											
41/52	488	447	160	31	A	13·9	8·2	7·0	5·7	4·9	3·6	2·7
(0·05 *I*)					C	10·9	6·6	5·4	4·5	3·5	2·7	1·8
					D	10·8	6·7	5·5	4·5	3·2	2·1	1·5
6/51	415	401	57	27	A	9·5	5·4			3·7	2·7	
(0·1 *I*)					C	9·3	4·8			3·5	2·5	
					D	9·4	4·2			3·2	2·1	
49/52	493	454	93	24	A	7·6		4·7		3·6	2·8	
(0·2 *I*)					C	7·1		4·2		3·3	2·5	
					D	7·4		3·9		3·0	2·3	

that of groups I and II. On the basis of isoelectric points Jacob (1947) has suggested that group II may represent myosin.

Mobility determinations. In all these analyses, but especially at 0·05 and 0·1 *I*, the ascending and descending diagrams showed a pronounced non-enantiography, and the stationary boundaries contribute up to 30 % of the total area of the tracings on the ascending side and 5–7 % on the descending side. Mobilities on the ascending side were consequently appreciably higher than on the descending side, the difference being particularly large with 6 and 7. The boundaries on the ascending side were always sharp, but on the descending side considerable spreading and overlapping of adjacent boundaries occurred, so that in some cases accurate mobility determinations could not be made. In order to use the ascending boundaries for mobility determinations the correction derived by Longsworth & MacInnes (1940) was applied where the traced areas of the ascending and descending diagrams were similar enough to justify it. The observed mobility of any component, j, on the ascending side is corrected by the factor

$$\frac{A_{ja}+A_\epsilon(V_{jd}/V_{ja})}{A_{jd}+A_\epsilon},$$

where A_{ja}, A_{jd} and A_ϵ are the traced areas of the ascending j boundary, descending j boundary and ϵ boundary, respectively. V_{ja} and V_{jd} are the volumes swept through by the ascending and descending j boundaries, respectively. This correction is approximate only since it takes no account of pH, conductivity and viscosity changes at each boundary, but the general agreement between the descending and corrected ascending mobilities suggests that it is reliable. Table 5 gives three examples of the application of the correction.

In general, the mobilities of the slowest components tend to be undercorrected. The results of several experiments at 0·05, 0·1 and 0·2 *I* are given in Table 6. In these experiments there appears to be no correlation between mobility and electrical field strength.

Table 6. *Mobilities of codling muscle components at* pH 7·5

(M, Arithmetical mean of corrected ascending and descending mobilities. S.D., standard deviation = $\sqrt{[\Sigma d^2/(n-1)]}$.)

I	Electrical field (V./cm.)	No. of experiments		Mobility (10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$) 1	2	3	4	5	6	7
0·05	4·0–7·5	10	M	10·7	6·7	5·6	4·4	3·6	2·6	2·0
			S.D.	0·45	0·36	0·37	0·16	0·34	0·41	0·35
0·1	3·2–9·0	4	M	8·9	4·5	3·5		3·0	2·1	
			S.D.	0·46	0·63	0·59		0·45	0·41	
0·2	3·3–4·7	5	M	7·1	3·8			3·1	2·3	
			S.D.	0·51	0·40			0·26	0·22	

The number of experiments is sufficiently large to show graphically the dependence of mobility upon ionic strength (Fig. 5). For the faster components 1 and 2, and possibly 3 and 4, the dependence resembles that found by Tiselius & Svensson (1940) for ovalbumin and can be satisfactorily explained on the basis of the Debye-Hückel-Henry theory. The mobilities of 5 and 6 are, however, greater at 0·2I than at 0·05 or 0·1I. This deviation may be due to the change from a purely phosphate buffer at 0·05 and 0·1I to a potassium chloride-phosphate buffer at 0·2I; Davis & Cohn (1939) observed that pronounced effects on electrophoretic mobility were brought about by changes in ionic environment.

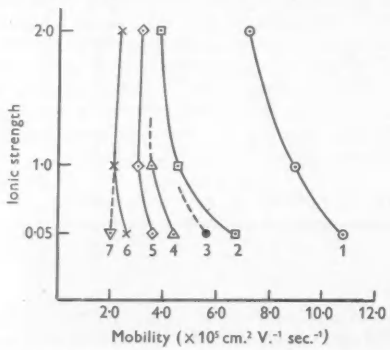


Fig. 5. Influence of ionic strength on electrophoretic mobility of codling skeletal muscle constituents.

The myogen and globulin X fractions. On dialysis for 2–3 days of a 0·05I extract to a conductivity of $3 \cdot 10^{-5}$ Ω^{-1} cm.$^{-1}$, about one-third of the protein precipitated and could be easily separated by centrifugation. This insoluble fraction corresponds to the globulin X of Weber & Meyer (1933). After washing with distilled water, dispersion in half the original volume of 0·05I phosphate buffer, pH 7·5, and dialysis to equilibrium against the same buffer, only about one-third of the globulin X could be brought into solution again. The insoluble portion was removed completely only by centrifuging at 20 000 *g*. A little more of the insoluble portion was soluble in 0·3 or 0·5I potassium chloride solution, but it undoubtedly consisted mainly of irreversibly denatured protein. The portion which was soluble in

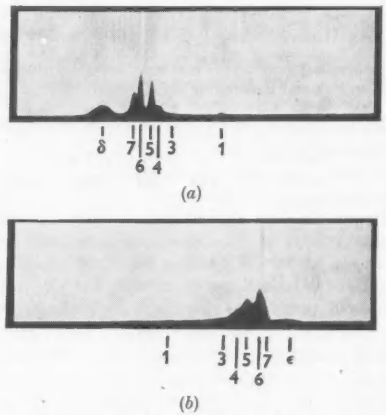


Fig. 6. Myogen fraction of codling skeletal muscle. (*a*) ascending limb; (*b*) descending limb. Exp. 56/52: 0·05I; pH 7·62; field strength, 6·23 V./cm.; duration of electrophoresis, 4080 sec.

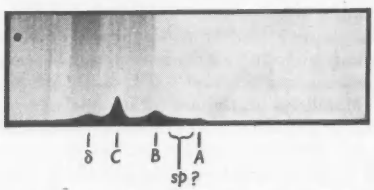


Fig. 7. Globulin X fraction of codling skeletal muscle. Ascending limb. Exp. 54/52: 0·05I; pH 7·61; field strength, 6·34 V./cm.; duration of electrophoresis, 3600 sec.

0·05I, and the completely water-soluble fraction (myogen) after equilibrium dialysis against 0·05I phosphate buffer, pH 7·5, gave the electrophoresis diagrams shown in Figs. 6 and 7.

Table 7. *Composition and mobilities of the myogen fraction* 0·05 I phosphate*, pH 7·5

	1	*sp*	3	4	5	6	7
% of total area†	1·4	3·4	2·4	9·6	37·6	33·1	12·4
Mobility 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$†	7·2–10·3	—	6·0	4·5	3·6	2·6	2·2

* Average N content: 2·00 mg./ml. † Average of four experiments.

The myogen diagram is similar to that of the original extract with the exception that 2 is undetectable. The components can be identified almost exactly by their mobilities, apart from 1, whose mobility varied from 7·2 to $10{\cdot}3 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ in four experiments (Table 7).

An examination of the average composition of the myogen fraction (Table 7) and of the average protein contents before and after complete removal of salts by dialysis reveals that the absolute amounts of all the components, except perhaps 5, have decreased. Jacob (1947) observed a similar effect with rabbit-muscle extracts dialysed against distilled water, and noted the parallel with the euglobulins of blood serum (Raffel, Pait & Terry, 1940; Svensson, 1941).

The globulin X diagrams show three diffuse but quite distinct components *A*, *B* and *C* whose mobilities (Table 8) correspond to 1, 3 and 7, respectively. However, the proportionate losses of all the components on dialysis and the absolute amounts of *A*, *B* and *C* (Table 8) indicate that *B* and *C* partially represent 2 and 4, and 6, respectively. Also, the losses of all the components, except perhaps 7, cannot be accounted for by the absolute amounts calculated from the globulin X diagram, and therefore nearly all the components must suffer some denaturation. The duration of dialysis cannot wholely explain this denaturation, since only small amounts of protein are precipitated from normal 0·05 *I* extracts on prolonged dialysis against 0·05 *I* phosphate buffer.

Table 8. *Composition and mobilities of the globulin X fraction* 0·05 I phosphate*, pH 7·5

	A	*sp*?	B	C
% of total area†	5·7	6·4	31·0	56·9
Mobility. 10^5cm.^{2}V.$^{-1}$sec.$^{-1}$†	10·6	—	6·0	2·1

* Average of two experiments.
† Average N content: 0·90 mg./ml. (≡0·45 mg./ml. in Tables 1 and 7).

DISCUSSION

Whereas rabbit muscle proteins can be analysed satisfactorily by electrophoresis at 0·15 *I*, the present work has shown that lower ionic strengths must be used in order to resolve the components of codling muscle extracts satisfactorily. Extracts of codling muscle at 0·15 *I* were intermediate in character between those at 0·1 and 0·2 *I*, and showed almost as little resolution as extracts at 0·2 *I*. Good resolution was obtained at 0·05 *I*, and this ionic strength is being used in routine work on fish muscle proteins, although such low ionic strengths are disadvantageous for quantitative electrophoretic analysis because of boundary anomalies, especially at relatively high protein concentrations. Because of this, the analyses recorded here are not necessarily absolute, although useful for comparison. Even at 0·1 *I*, the boundary anomalies and asymmetries were almost as great as at 0·05 *I*.

The electrophoretic diagrams of extracts of codling muscle at low ionic strengths, although of equal complexity, show the same general characteristics as those of rabbit or frog muscle. The appearance of the faster components and components of intermediate mobility of codling suggests that they may be identified with Jacob's components *h*, *j*, k_1 and k_2, and Bosch's components 2, 3, 4 and 5, although the mobilities of the fish proteins are higher. The value and disposition of the mobilities of the slowest group of codling components, however, are sufficiently different to establish their non-identity with the members of the corresponding group of rabbit components. It would be interesting to work out this difference in terms of the muscle enzymes of rabbit and codling.

Jacob (1947) has attempted to correlate the electrophoretic components of rabbit muscle with the classical muscle proteins, and in general his conclusions apply to codling muscle. It is probable that group III represents myoalbumin; quantitatively this means that it is present as less than 1 % of the total codling muscle proteins (cf. Hamoir, 1951*b*). Dyer *et al.* (1950) found about 7 % of myoalbumin in cod muscle using Bate-Smith's (1937) method, but attempts to estimate this fraction (Connell, unpublished) by the same method failed to reveal significant amounts of acid-stable protein in codling. The globulin X of codling muscle is electrophoretically almost as complex as the total non-myosin proteins, and it is evident that the characterization of this type of fraction by simple loss of solubility on removal of salts is far from adequate.

SUMMARY

1. Extracts of codling skeletal muscle at 0·05, 0·1 and 0·2 *I*, pH 7·5, have been examined in the Tiselius electrophoresis apparatus.

2. Extracts at 0·05 *I*, pH 7·5, proved to be the most informative, and revealed the presence of at

least seven components which may be divided into Jacob's groups I, II and III.

3. Whereas groups II and III of codling bear similarities to the corresponding groups of rabbit, group I is quite different.

4. Group II appears to be particularly significant in relation to the denaturation of these protein mixtures.

5. The mobilities of codling muscle proteins are appreciably greater than those of rabbit.

6. The influence of ionic strength on the mobilities of these proteins has been examined.

7. On dialysis of a 0·05 *I* extract against distilled water, component 2 precipitates completely, and all the components, except perhaps 5, suffer a partial loss of solubility. The precipitate of globulin X is only partially re-soluble; the soluble portion shows three main components which on the basis of mobility can be related to components in the original extract. However, estimates of composition reveal that the relationship is complex.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Lipids of Fish

1. CONTENT AND CONDITION OF LIPIDS IN THE FLESH OF THE HADDOCK (*GADUS AEGLEFINUS*)

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(*Received* 1 *September* 1952)

The series of papers which it is hoped to present under the general title 'The Lipids of Fish' will describe studies preparatory to investigations of the lipoprotein constituents of fish tissues. From the outset, therefore, considerable emphasis has been placed on the relative ease with which any particular lipid may be extracted from the tissue, since much of the evidence for the existence of lipid-protein complexes is based on the occurrence of so-called 'bound' lipids, i.e. lipids which cannot be extracted from the fresh tissue by solvents in which they are normally readily soluble. At the same time, segregation of constituents according to ease of extraction may well assist in the separation of the extremely complex mixture of lipids present in the tissue.

The flesh of the haddock has been chosen as the most suitable raw material for the present series of studies, since it is quite free from adipose tissue, it can be obtained absolutely fresh in virtually unlimited quantity from this laboratory's own fishing vessel, and, the haddock being a small fish, it offers the advantage that lipid samples are usually representative of a very large number of individuals.

The present paper reports the results of successive extractions of haddock flesh with a range of solvents.

EXPERIMENTAL

A lipid is generally considered to be 'free' when it can be extracted from the tissue with a solvent which does not denature protein, such as, for example, light petroleum and ethyl ether. Extraction after treatment with such a solvent as ethanol is considered to yield much of the lipid originally linked to protein.

Finely minced haddock flesh exhaustively extracted with light petroleum, either by grinding with sand or by use of a Waring Blendor, yielded about 0·1% of lipid. (All percentages are w/w.) If the extracted tissue was then dried under vacuum and re-extracted with light petroleum, a further trace (about 0·02%) of lipid could be recovered. Subsequent extraction with ethyl ether yielded about 0·05% of additional lipid. Thus the total 'free lipid' amounted to about 0·17%. There were, however, considerable variations from fish to fish and the range encountered in a considerable number of experiments was from 0·06 to 0·13%, 0·02 to 0·03% and 0·03 to 0·11% for the three extracts respectively. The I_2 values of these extracts varied even more widely; from 82 to 188, 13 to 90 and 28 to 132 respectively. Such great individual variations emphasize the desirability of obtaining lipid samples representative of a large number of individuals.

If the exhausted tissue was next extracted with cold ethanol, a further yield of about 0·1% (range 0·06–0·15%) of the original fresh tissue weight was obtained. Hot ethanol, on the other hand, extracted about 0·5% of lipid together with an equal quantity of non-lipid substances.

Tissue exhausted with cold ethanol yielded a further 0·15% or so of lipid to cold $CHCl_3$, but tissue exhausted by hot ethanol yielded only about 0·05%, even to hot $CHCl_3$. The ethanol extracts had I_2 values of about 40 and the $CHCl_3$ extracts about 60, although again there were considerable variations from fish to fish.

The final exhausted tissue still contained lipid, as could be shown by acid hydrolysis of the protein followed by recovery of the freed fatty material. If only cold solvents had been used, a further 0·4–0·6% of lipid could be recovered after acid treatment, but if hot ethanol and $CHCl_3$ had been used only about 0·1% of additional lipid could be obtained after hydrolysis. This lipid liberated by hydrolysis was presumably largely in the form of free fatty acids and its I_2 value ranged from 90 to 164.

The total lipid content of haddock flesh thus extracted ranged from 0·75 to 1·1%. In order to prepare large enough samples of lipid for detailed investigation a different series of solvents was used after preliminary studies of their relative effectiveness.

A total of 42 kg. of minced haddock flesh was extracted in batches of 7 kg. with large volumes of acetone at room temperature (approx. 15°), through a total of nine successive extractions for each batch. The first acetone extract of each batch consisted mainly of water-soluble, light petroleum-insoluble, non-lipid matter (about 2% of the tissue) and had a marked smell of NH_3, even when obtained from material extracted at sea from fish immediately after death. Nevertheless, these first extracts did contain some true lipid matter. The ninth acetone extract in all cases contained so little lipid that it was clear that almost all lipid available to this solvent had been removed. The combined extracts 2–9 were evaporated, the residue taken up in light petroleum to remove traces of non-lipid matter, the solution combined with the petroleum extract of acetone-extract 1, and the whole evaporated to yield 241 g. of lipid (=0·57%). It may be noted that acetone has been found to have an effect comparable to that of ethanol in freeing 'bound' lipid (Delage, 1935). The exhausted tissue was next extracted at room temperature with large volumes of ethanol/ether (3:1, v/v), again through a total of nine extractions, the last extract containing negligible amounts of lipid. The total lipid obtained in this fraction was 94 g.=0·22%. The residual tissue was then extracted nine times with a boiling benzene/ethanol (1:2, v/v) mixture allowing 30 min. boiling at each extraction, to give a total of 79 g. (0·19%) of lipid; subsequent similar extraction with a boiling $CHCl_3$/methanol (1:1, v/v) mixture gave 103 g. (0·24%) and, lastly, extraction with pyridine at 100° gave 65 g. (0·15%) of lipid. The total lipid obtained was thus 1·37% of the original tissue, compared with the range 0·75–1·1% obtained in the preliminary experiments. Acid hydrolysis of the final exhausted tissue showed that it contained only negligible traces of residual lipid.

DISCUSSION

Little appears to be known concerning the lipids of haddock flesh, or indeed of fish muscle tissue in general, work on the lipids of fish having been primarily concerned with adipose tissue whether mixed with the skeletal musculature or not. Bähr & Wille (1931) reported on the 'lecithin' content of fish flesh, including haddock flesh, but their method of extraction of the air-dried flesh with an ethanol/benzene mixture followed by determination of phosphorus in the extract and calculation of all phosphorus found as lecithin phosphorus is open to criticism. They showed, however, that fish flesh varied widely in 'lecithin' so determined, according to species, age, season of year, and stage of maturity. Thus haddock flesh in June contained 0·17% lecithin, whereas comparable material in October contained 0·48%. Kaucher, Galbraith, Button & Williams (1943) reported that codfish muscle contained a total of 9·47% lipid on a dry-weight basis, which would be about 2·5% on the wet tissue. This was extracted from freeze-dried tissue by hot ethanol followed by ethyl ether. Cod and haddock belong to the same genus, and it is rather surprising that the former should contain nearly twice as much lipid as the latter. The fractionation of these lipids by Kaucher *et al.* is discussed in the following paper.

The only large fat depot in the haddock is the liver. It would appear from the present results of extraction with light petroleum and ether that the major part of the lipids of haddock skeletal muscle are in 'bound' form. There seem to be several types of linkage holding this bound lipid, of varying ease of rupture, e.g. some lipid is freed by alcohol, some still bound and liberated after acid hydrolysis. The results of the large-scale extraction, although giving

a qualitatively and quantitatively different picture, also suggest a variety of linkages between the lipids and the protein of the flesh.

The various large-scale extracts revealed considerable differences in appearance, consistency, etc., and it seems probable that they have been separated not only according to the relative strength of their linkages to protein, but also according to their solubility properties. A considerable overlap in their composition would, therefore, be expected. The following paper records the results of investigation of two sub-fractions of the first (acetone) extract.

SUMMARY

1. Haddock flesh contains a total of about 1% lipid, but only about 0·1–0·2% of 'free' lipid, the rest being probably 'bound' to protein.

2. Extraction with a series of solvents suggests the presence of more than one type of linkage between lipid and protein although the picture is complicated by probable differences in solubility of the various lipid constituents.

This work has been carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Lipids of Fish

2. THE ACETONE-SOLUBLE LIPIDS OF THE FLESH OF THE HADDOCK

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(*Received* 1 *September* 1952)

In the previous paper (Lovern, 1953) the preparation of a series of lipid extracts from the flesh of the haddock (*Gadus aeglefinus*) was described. The first of these consisted of material extracted from the tissue by acetone and soluble in light petroleum. It is well known that in the presence of such soluble lipids as triglycerides, sterols and sterol esters, acetone will dissolve other lipids, e.g. phospholipins, which alone are insoluble in acetone. This particular extract contained 2·6% phosphorus and 1·6% nitrogen. There has been controversy, particularly in earlier work, about the occurrence of phospholipins genuinely soluble in acetone, i.e. soluble in the absence of such lipids as fats and sterols. MacLean (1914) showed that such phospholipins do occur but can be completely precipitated from acetone in the presence of traces of electrolytes. Removal of impurities, presumably electrolytes, can render an originally acetone-insoluble phospholipin soluble in acetone (MacLean, 1909*a*). In the purification of phospholipin preparations by repeated precipitation from ether or light petroleum solution by a large excess of acetone, many workers add an electrolyte, commonly magnesium chloride. There are, however, objections that such a procedure gives a variable yield according to the amount of electrolyte added, and that the nature of the phospholipin is altered in the process, e.g. the proportion of ether-insoluble phospholipin increases with increasing amount of electrolyte (Sinclair & Dolan, 1942). Cahn, Houget & Agid (1949) discussed in detail the relative merits of precipitation with acetone in the presence or absence of added electrolytes and showed that electrolytes also precipitate large proportions of non-lipid contaminants.

We considered it desirable to examine separately the phospholipin fraction genuinely soluble in acetone in the absence of added electrolytes and that present in the total acetone extract but precipitable when accompanying non-phosphatidic lipids had been removed. The technique of counter-current distribution, used in our earlier studies on lipid separation (Lovern, 1952; Olley, 1953), was employed to examine the fractions obtained by acetone precipitation. The non-phosphatidic lipids, together with the acetone-soluble phospholipins, form the subject of the present paper.

Part of this work was presented at the Second International Congress of Biochemistry, Paris, 1952.

EXPERIMENTAL

Analytical methods

Choline and serine plus ethanolamine were estimated as described by Lovern (1952), N, P and plasmal as described by Olley (1953), glycerophosphate as in Olley & Blewett (1950), total cholesterol by the method of Rose, Schattner & Exton (1941) applied direct to the lipid; free cholsterol was estimated by the same method applied to the digitonide, the latter being precipitated according to the procedure of Sobel & Mayer (1945) but retaining the acetone/ether wash of the original Schoenheimer & Sperry (1934) method. Glycerol was determined by Whyte's (1946) method scaled down 10 times. Fatty acid analysis by ester distillation was essentially as described by Guha, Hilditch & Lovern (1930), with some later modifications (cf. Lovern, 1934, 1942).

All lipid materials were stored under N_2 at $-30°$ until required for analysis.

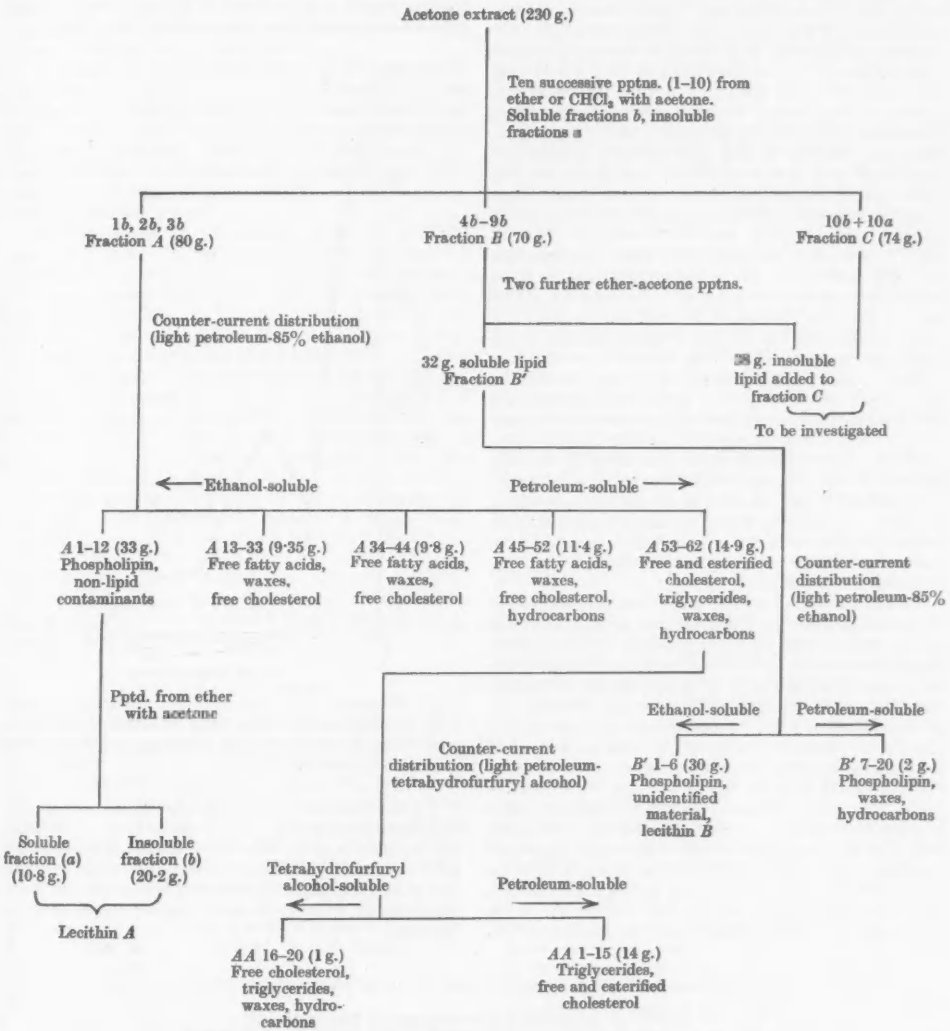
Acetone extract (230 g.)
Ten successive pptns. (1–10) from ether or CHCl3 with acetone. Soluble fractions b, insoluble fractions a
1b, 2b, 3b Fraction A (80 g.)
4b–9b Fraction B (70 g.)
10b + 10a Fraction C (74 g.)
Two further ether-acetone pptns.
Counter-current distribution (light petroleum-85% ethanol)
32 g. soluble lipid Fraction B′
38 g. insoluble lipid added to fraction C
To be investigated
Ethanol-soluble
Petroleum-soluble
A 1–12 (33 g.) Phospholipin, non-lipid contaminants
A 13–33 (9·35 g.) Free fatty acids, waxes, free cholesterol
A 34–44 (9·8 g.) Free fatty acids, waxes, free cholesterol
A 45–52 (11·4 g.) Free fatty acids, waxes, free cholesterol, hydrocarbons
A 53–62 (14·9 g.) Free and esterified cholesterol, triglycerides, waxes, hydrocarbons
Counter-current distribution (light petroleum-85% ethanol)
Pptd. from ether with acetone
Ethanol-soluble
Petroleum-soluble
Counter-current distribution (light petroleum-tetrahydrofurfuryl alcohol)
B′ 1–6 (30 g.) Phospholipin, unidentified material, lecithin B
B′ 7–20 (2 g.) Phospholipin, waxes, hydrocarbons
Soluble fraction (a) (10·8 g.)
Insoluble fraction (b) (20·2 g.)
Lecithin A
Tetrahydrofurfuryl alcohol-soluble
Petroleum-soluble
AA 16–20 (1 g.) Free cholesterol, triglycerides, waxes, hydrocarbons
AA 1–15 (14 g.) Triglycerides, free and esterified cholesterol

Fig. 1. Schematic summary of the fractionation of the acetone extract of the flesh of the haddock.

Fractionation with acetone

A summary of the fractionation procedure is shown in Fig. 1.

Acetone-extract (about 230 g.) of haddock flesh prepared according to Lovern (1953) was mixed with 300 ml. of ether to a viscous solution and poured slowly with constant stirring into 6·6 l. of acetone at 0°. There was an immediate precipitation of a thick gum and on standing overnight at 0° a further precipitation of a flocculent nature. The total insoluble material (1*a*) weighed 185 g. and the soluble (1*b*) 44 g. Fraction 1*b* was made up to 100 ml. with ether and poured into 2 l. of acetone at 0°, but proved entirely soluble. It contained 1·0% P, no N and gave a strong reaction for cholesterol. Fraction 1*a* was again taken up in ether and precipitated with acetone as before. In this case the first appearance of the precipitate was flocculent and it later became gummy. Yield of insoluble fraction (2*a*) 153 g. and of soluble fraction (2*b*) 32 g. Fraction 2*b* contained 2·1% P, 0·8% N and much cholesterol. The same reprecipitation procedure applied to 2*a* gave a flocculent precipitate which settled overnight to a spongy mass. This precipitate, unlike the previous ones, proved to be insoluble in ether, but readily soluble in $CHCl_3$. All subsequent precipitates in this acetone-fractionation series were insoluble in ether but soluble in $CHCl_3$, which was, therefore, used instead. We have repeatedly observed that when crude phospholipin mixtures are fractionated, either by precipitation methods or by counter-current procedures, certain fractions progressively become less soluble or even abruptly virtually insoluble in ether. We feel that this is probably due to the previously mentioned effects of accompanying electrolytes, or other contaminants, which are being concentrated in certain fractions, and is similar to the effects on solubility in acetone and to the effect on ether solubility noted by Sinclair & Dolan (1942).

Fraction 2*a* yielded 142·5 g. of insoluble (3*a*) and 10·5 g. of soluble material (3*b*). The latter gave a definite reaction for cholesterol. Further reprecipitations of the acetone-insoluble portion under the same conditions of dilution and temperature yielded a series of acetone-soluble fractions as illustrated in Fig. 2. (Insoluble and soluble fractions are given the suffixes *a* and *b*.) Fraction 4*b* no longer gave a reaction for cholesterol, and it and all subsequent ones had a typically phosphatidic appearance, in contrast to fractions 1*b* and 2*b* which were oily liquids, 3*b* being intermediate.

Fig. 2 illustrates the marked effect of electrolytes or other effective contaminants on solubility in acetone. Only on the basis of selective removal or concentration of such agents is it possible to explain the rise from the minimum at 3*b* to the maximum at 5*b*. The levelling out of the weight curve for the last three fractions suggests that this value of about 5 g. represents the solubility of the 'insoluble' fraction in 6·6 l. of acetone (containing 200 ml. of $CHCl_3$) at 0°. Fraction 10*b* was accordingly combined with the final acetone-insoluble fraction 10*a*, giving 74 g. of material (fraction *C*), fractions 1*b*–3*b* were combined to give 80 g. (fraction *A*) of material containing, presumably, all the non-phosphatic lipid (e.g. all the cholesterol), together with some phospholipin, and fractions 4*b*–9*b* were combined to give 70 g. of a fraction (*B*) consisting mainly of acetone-soluble phospholipins.

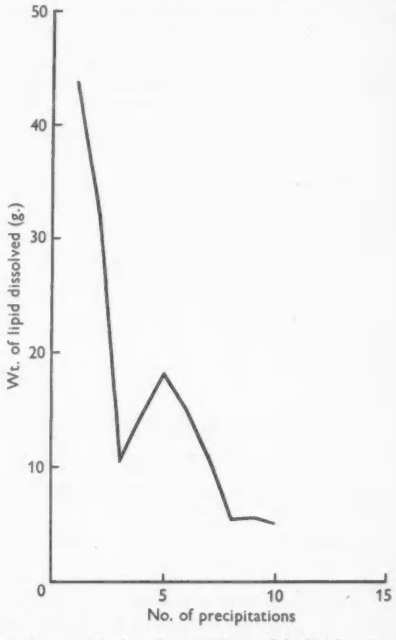

Fig. 2. Reprecipitation from acetone of the lipids extracted by acetone from haddock flesh. Total initial wt. 224 g. Curve shows weight of lipid remaining in solution after each precipitation.

A general comparison of the phospholipins contained in these three fractions is given in Table 1. It should be noted that for fraction *A* the data relate only to the phospholipin portion separated by counter-current distribution from the non-phosphatidic lipids as described below, whereas it was assumed that fractions *B* and *C* consist mainly of phospholipins.

Table 1. *Analytical data for fractions B and C and the crude phospholipin portion of fraction A*

(Values are percentages of constituents in the fractions.)

Fraction	N (%)	P (%)	N:P ratio (atomic)	Choline (%)	Serine + ethanolamine *N* (%)	Plasmal (%)
A (1–12)*	2·32	3·25	1·58	11·2	Nil	Trace
B	1·80	3·05	1·31	10·1	0·17	4·7
C	2·21	3·34	1·47	6·2	0·13	2·0

* Phospholipin portion, see p. 131.

Table 1 shows that all preparations contained too high an N:P ratio for monoaminophospholipins and that neither repeated precipitation from acetone nor counter-current distribution in the case of fraction *A* had removed the excess N. The fractions also contain considerable uncharacterized lipid, the recognized phospholipin bases and P contents being less than required for a mixture of the classical phospholipins. The greatest discrepancy in respect of nitrogenous bases is shown by fraction *C*.

In view of the unexpected course of the acetone-precipitation curve in Fig. 2, and as we have been unable to find any comparable published experiments, it may be mentioned that prior to carrying out the present series of studies we had conducted exploratory work with an exactly comparable series of haddock flesh lipid extracts, prepared some 2 years earlier. This preliminary work gave the same form of acetone-precipitation curve.

Since the solubility of fraction *C* in acetone at 0° was apparently about 5 g./6·6 l., it seemed that fractions 1–9 might each be contaminated with about 5 g. of *C* and hence fraction *B* might contain about 30 g. of material really belonging to fraction *C*. Fraction *B* was accordingly dissolved in 250 ml. ether and precipitated in 6 l. of acetone at 0°, yielding 24 g. soluble and 46 g. insoluble lipid. The latter was a thick gum probably occluding some soluble lipid, and hence was reprecipitated with 100 ml. of ether and 4 l. of acetone, yielding an additional 8 g. soluble and 38 g. insoluble lipid. A total of 10 l. of acetone could dissolve about 7·5 g. of fraction *C*, so that the predicted yield of insoluble matter was about 23 g. instead of the 38 g. actually obtained, again illustrating the complexity of the factors affecting the solubility of phospholipins in acetone. As shown later, fraction *A* also contained much phospholipin really belonging to fraction *C*. For the present examination, the 38 g. of insoluble lipid obtained from *B* were added to *C*, leaving 32 g. of the final fraction *B'*. *B'* contained 2·1% N before hydrolysis, 1·8% water-soluble N after hydrolysis, 9·2% choline, 2·0% plasmal and 3·3% P.

Analysis of fractions

Fractions *A* and *B'* were submitted to counter-current distribution between 85% (v/v) ethanol and light petroleum (b.p. 40–60°), using conical flasks as units, as described by Lovern (1952). When conducted manually this operation is particularly flexible, and the sequence of transfers can be modified at any stage according to the appearance of both phases at various positions in the chain of units, or the progress of elution from the end of the chain. Periodical emulsification, usually late in the sequence, was again observed (cf. Lovern, 1952). Emulsified material was separated centrifugally, and a marked tendency was noted for such material to re-emulsify on each transfer right through the series.

Fraction A

Fraction *A* (80 g.) was added to the first of thirty flasks containing 400 ml. of light petroleum and successive batches of 400 ml. of 85% ethanol were passed down the chain and run off as a series of fractions. By the fifteenth such fraction the yield had become very low and the appearance of the petroleum in flasks 19–30 suggested that these flasks contained almost no lipid, hence they were withdrawn from the system and the contents of each separately evaporated. The fifteen ethanol fractions run off are numbered 1–15 in Fig. 3 and flasks 30–19 of light petroleum yielded fractions 16–27 in Fig. 3. Seventeen further batches of ethanol were then run through the remaining eighteen flasks of light petroleum (1–18), and these yielded fractions 28–44 in Fig. 3. Finally the light petroleum in flasks 18–1 was evaporated to yield fractions 45–62 in Fig. 3. The sequence of counter-current events is thus arranged in Fig. 3 to run continuously according to the tendency towards one or the other phase, vertical dotted lines indicating where the curves are really discontinuous. Certain fractions were analysed for N, P, choline, free and esterified cholesterol, the results being shown in Fig. 3.

Fractions were pooled according to analyses, appearance and weight yield. Fractions 1–12 were combined to include virtually all of the phospholipin, fractions 13–33 to give a fraction relatively sterol free, fractions 34–44 a mixed fraction rich in free sterol, fractions 45–52 a main free sterol fraction, and fractions 53–62 to include all esterified cholesterol. (From other experiments we know that in counter-current distribution with these solvents, triglycerides largely accompany esterified cholesterol and should, therefore, also be almost exclusively contained in fractions 53–62. As will be shown below, such was the case.)

Fractions A 1–12. The general analysis of fractions *A* 1–12 is given in Table 1. In an attempt to remove the excess N, this fraction (about 33 g.) was submitted to counter-current separation in a series of four flasks between light petroleum and 20% (v/v) ethanol (400 ml. each phase). Three ethanol fractions were eluted and contained only 0·253 g. in the first eluate and 0·185 g. in the second and third combined. These fractions contained 6·9 and 6·2% N and 0·4 and 0·7% P respectively. They were only partially soluble in ether but readily soluble in water. The removal of larger amounts of N-rich material was attempted in seven flasks between light petroleum and 50% (v/v) ethanol, but abandoned because of severe emulsification (not found with 20% ethanol). The small yield of ethanol phase recovered contained 0·13 g. of material with 5·7% N and 1·9% P. The experience of Olley (1953) suggested that a very high lipid concentration might prove effective and hence the material (approx. 32 g.) was dissolved in 100 ml. of light petroleum and shaken with 100 ml. of 85% (v/v) ethanol. The two phases proved completely miscible, although in previous work with phospholipins we have used such concentrations. The volumes of both phases were doubled, when ready separation occurred. A series of seven ethanol fractions was run through nine batches of light petroleum, but examination of the fractions showed no helpful removal of excess N; thus fractions 1, 2 and 5 of the petroleum phase contained only 1·4, 1·9 and 2·0% N respectively, the excess N accompanying the main weight of material into the ethanol. The combined petroleum fractions (5·8% of the total) were taken up in ether and washed with dilute aqueous Na_2CO_3, since free fatty acids were found in many other fractions (see below). This material yielded 0·6 g. free fatty acid and 1·1 g. neutral substance.

The ethanol-phase crude phospholipin fraction contained 2·3% N, 3·2% P (N:P ratio, 1·6), 11·2% choline, 0·07% α-amino-acid N and 0·3% N liberated as NH_3 by addition of alkali to an acid hydrolysate.

Since non-phosphatidic lipids had now been largely removed, a test was made of the acetone solubility properties of this fraction. About 30 g. was dissolved in 100 ml. of ether and poured into 1500 ml. of acetone at 0°, giving 35% soluble (fraction *a*) and 65% insoluble lipid (fraction *b*). This again illustrates the effect of the presence of other lipids

or of a particular concentration of electrolytes. Fraction (*a*) proved devoid of free fatty acids (fraction (*b*) would naturally be so), and the 1·1 g. of neutral product from the petroleum phase was combined with it. Fractions (*a*) and (*b*) contained 2·4 and 2·6% N and 3·3 and 3·8% P respectively. They were separately hydrolysed by refluxing for 2 hr. with 0·5 N-ethanolic KOH. After addition of water the soap solutions were extracted with ether giving 8·8 and 3·6% unsaponifiable matter respectively. This had I_2 values of 159 and 222 and, after acetylation, saponification equivalents of 484 and 300. Fractions (*a*) and (*b*) further yielded 9·5 and 12·6% choline and 56·5 and 60·4% fatty acids respectively, the latter having I_2 values of 276 and 243. The two lots of fatty acids were combined, giving enough material for a quantitative analysis by ester distillation, the results of which are given in Table 2.

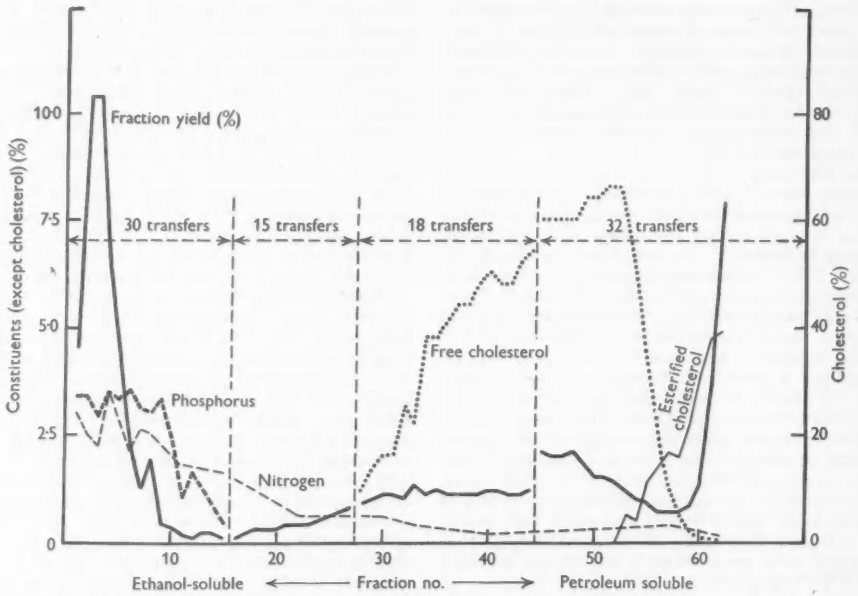

Fig. 3. Haddock flesh lipids. Counter-current distribution of acetone-soluble fraction *A* between light petroleum and 85% (v/v) ethanol. Fifteen successive batches of ethanol (fractions 1–15) passed through a chain of thirty flasks containing light petroleum. Flasks 30–19 withdrawn, contents giving fractions 16–27. A further seventeen batches of ethanol (fractions 28–44) passed through the remaining eighteen flasks, the contents of flasks 18–1 finally becoming fractions 45–62. Curves for yield, free and esterified cholesterol pass through experimental values for every fraction. Experimental values for fractions 1–11, 15, 22, 30, 36, 40, 57 and 62 on nitrogen curve and for fractions 1–12 and 15 on phosphorus curve. Zero phosphorus values found on fractions 22, 30, 33, 36 and 40. Free cholesterol content of pooled fractions 16–27 = 8%.

Table 2. *Fatty acid composition of certain lipids of the haddock*

(Weights as % of total fraction.)

	Saturated acids				Unsaturated acids					
Material	C_{14}	C_{16}	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Lecithin '*A*' (flesh)	—	13·5	5·7	2·9	—	—	12·6 (5·4)*	33·7 (8·0)	27·3 (12·0)	4·3
Lecithin '*B*' (flesh)	—	16·4	4·0	2·1	—	—	5·4 (5·5)	42·2 (8·0)	29·9 (11·0)	—
Free fatty acids (flesh)	—	12·1	7·3	3·7	—	—	20·6 (4·0)	37·4 (7·5)	18·9 (10·0)	Trace
Depot fat† (liver)	4·3	14·1	0·3	—	0·5	12·4 (2·0)	30·5 (2·6)	29·3 (5·9)	8·6 (7·3)	—

* Figures in parentheses show average unsaturation as lack of hydrogen atoms.
† Lovern (1932).

In view of the presence of uncharacterized N, it may be noted that after alkaline hydrolysis only 1·3 and 1·4% N could be found in the aqueous phase of (*a*) and (*b*), in contrast to the 2·4 and 2·6% before hydrolysis. Part of the loss may correspond to the 0·3% N released as NH_3 on adding alkali to an acid hydrolysate, but a further source of loss is N present in the fatty acids. Other workers (MacLean, 1909*a*, *b*; Trier, 1913*a*, *b*, *c*; Bülow & Page, 1932) have reported that fatty acids liberated by either alkali or acid hydrolysis of phospholipins contain about 0·1–0·5% N which cannot be removed by thorough washing or prolonged hydrolysis. Our fatty acids contained 0·2 and 0·5% for fractions (*a*) and (*b*) respectively. Trier (1913*a*) further found that fatty acids recovered after hydrolysis with $Ba(OH)_2$ contained P equal to 0·12% of the original lipid. We did not examine either fatty acids or unsaponifiable matter for P, but we did note that the aqueous solution after hydrolysis of (*a*) and (*b*) contained P equivalent to only 2·3 and 3·2% of the original lipids respectively, compared with 3·3 and 3·8% before hydrolysis. The glycerophosphate P in the solution was even less, being 2·0 and 2·4% respectively of the original lipid.

Fractions A 13–33. Fractions 13–33 (total weight 9·35 g.) gave 95% of ether-soluble lipid, which was a mobile liquid at room temperature, and 5% of ether-insoluble, water-soluble substance, which was discarded. The purified lipid (7·45 g.) was washed in ether solution with Na_2CO_3 solution, yielding 4·70 g. free fatty acids and 2·75 g. neutral material. The latter, an oily liquid at room temperature, was saponified with ethanolic KOH to yield 64·4% unsaponifiable matter and 38·6% fatty acids (I_2 val., 236·2). The unsaponifiable matter contained 48·7% cholesterol, hence the fraction contained 33·0% non-sterol unsaponifiable matter. It further contained 0·3% glycerol. Allowing for fatty acids combined with this, the percentage of fatty acid apparently combined with the 33% of non-sterol unsaponifiable matter is 35·6%. (All the cholesterol in this fraction is in the free state.) Such a close ratio strongly suggests a wax ester, with the unsaponifiable portion consisting of higher aliphatic alcohols. The amounts available were too small for satisfactory further examination.

Fractions A 34–44. Fractions 34–44 (total weight 9·77 g.) yielded 0·1 g. ether-insoluble impurity, which was soluble in water or $CHCl_3$. It was a light brown powder containing 0·8% N and 0·5% P. Hydrolysed with 0·5 N-ethanolic KOH it yielded 36·5% fatty acids (I_2 val., 99·0) and no unsaponifiable matter. The main portion of fractions 33–44 (8·93 g.) was crystallized from ethyl acetate to yield 2·5 g. cholesterol and 6·34 g. concentrate. The latter (6·06 g.) was worked up in a similar manner to fractions 13–33, giving 4·27 g. free fatty acids and 1·79 g. neutral product, which on saponification yielded 13·4% fatty acids (I_2 val., 174·6) and 55·9% unsaponifiable. The latter contained 76·3% cholesterol, hence the content of non-sterol unsaponifiable matter in the fraction was 13·2%. The glycerol content was 0·2%, hence fatty acids not present as triglycerides amount to 11·7%, again in close agreement for a wax ester with the amount of non-sterol unsaponifiable matter.

Fractions A 45–52. Fractions 45–52 (11·41 g. total weight) were entirely soluble in ether; 10·07 g. crystallized from ethyl acetate yielded 4·01 g. cholesterol and 6·06 g. concentrate. Treated in the same way as the preceding fractions 5·54 g. of this concentrate gave 2·17 g. free fatty acids and 3·37 g. neutral product which, on saponification, gave 12·2% fatty acids (I_2 val., 166·3) and 62·6% unsaponifiable, which itself contained 44·9% cholesterol. The glycerol content was 0·4%, hence fatty acids not present as triglycerides amount to 8·3%, to be compared with 34·5% non-sterol unsaponifiable matter. Here there is a considerable discrepancy compared with the requirements for wax esters, and it seems likely that types of unsaponifiable matter other than higher aliphatic alcohols, e.g. hydrocarbons, are also present.

Fractions A 53–62. Fractions 53–62 (total weight 14·94 g.) proved to contain 0·23 g. free fatty acids. The neutral material contained free and esterified cholesterol and presumably also triglycerides. An attempt was made to separate these by counter-current distribution between light petroleum and tetrahydrofurfuryl alcohol. Ethanol is not a sufficiently good solvent for cholesterol esters or triglycerides to move them at any useful rate through light petroleum. Unfortunately, although the new solvent pair gave very efficient separation of free and esterified cholesterol, it did not effectively separate the latter from triglycerides. Many other solvent pairs were tried without success, part of the difficulty, no doubt, arising from the large range of fatty acids present in both types of compound, giving very broad and overlapping bands. Of the twenty subfractions obtained by the use of tetrahydrofurfuryl alcohol, nos. 16–20 (those most preferentially soluble in the lower phase), amounting to 7% of the total, were the only ones containing no esterified cholesterol. They were pooled and found to contain 2·5% glycerol, 30% unsaponifiable matter other than cholesterol and yielded 39·8% fatty acids on hydrolysis. The excess of fatty acids (assuming average chain length C_{19}) over the requirements of triglycerides is 15·8%, hence the unsaponifiable matter was assumed to be about half higher aliphatic alcohols occurring as wax esters, and half hydrocarbon or similar substance.

An attempt to examine separately the fatty acid mixture of the cholesterol esters and of the triglycerides in the combined first fifteen subfractions by selective enzymic hydrolysis of the latter (Kelsey & Longenecker, 1941) was spoiled by accidental loss of most of the material. The combined material contained 3·3% glycerol, 33% esterified cholesterol and 6% free cholesterol. Again assuming an average fatty acid chain length of C_{19}, the content of cholesterol esters would be 58·3% and of triglycerides 33·6%, thus accounting with the 6% free cholesterol for 98% of the total.

The free fatty acids removed from the various fractions were combined and analysed by fractional distillation of the methyl esters, the results being given in Table 2. The occurrence of free fatty acids in a tissue extract is always suspect, since they may be a product of rapid post-mortem autolysis. Fairbairn (1945) has shown how rapid this may be in the case of liver phospholipins. The fish used in preparing these extracts had been preserved with ice for up to 18 hr. before extraction. To test this point, a sample of 10 kg. of haddock flesh was obtained from freshly killed fish at sea and minced directly into a large excess of acetone. Subsequent extraction was as before. The product was fractionated by acetone precipitation, separated by counter-current extraction between light petroleum and ethanol, and the phospholipin-free portion extracted with Na_2CO_3 solution. The yields of various lipids at all stages were comparable to those obtained in the main experiment, and a total of 3·4 g. of free fatty acids was obtained from the 10 kg. of fish. Bloor (1926) found free fatty acids amounting to about 2% of the total fatty acids in the lipids of ox heart.

The total composition of this first subfraction *A* of haddock flesh lipids has been derived by summation of the foregoing data and the results are given in Table 3. It has been assumed that combined fatty acid in excess of that directly accountable as triglyceride, esterified cholesterol or lecithin was present as wax esters. In all cases where there was evidence of such 'excess' fatty acid there was enough unsaponifiable matter other than cholesterol to support this assumption. The content of lecithin was based on the choline contents. 'Non-lipid impurity' was obtained by difference, 84% of it being concentrated in the lecithin fractions. It could be calculated that the 'impurity' in lecithin fractions (*a*) and (*b*) respectively contained 5·9 and 17·1% N and 4·5 and 10·9% P. An average chain length of 19 carbon atoms has been assumed for the fatty acids and unsaturation has been disregarded in making certain calculations, e.g. weight of cholesterol esters from weight of esterified cholesterol.

Table 3. *Component lipids of subfraction A of the acetone extract of haddock flesh* (%)

Lecithin	32·1	Wax esters	8·4
Free cholesterol	17·8	Triglycerides	6·8
Free fatty acids	17·7	Hydrocarbons, etc.	2·0
Cholesterol esters	9·8	Non-lipid impurities	5·4

Fraction B′

The counter-current analysis of fraction *B′* was made in a series of twenty flasks, using 150 ml. of each phase (light petroleum and 85% (v/v) ethanol) for about 32 g. lipid. An attempt to use only 100 ml. of each phase, in the light of Olley's (1953) experience, resulted in complete miscibility of the phases. Ethanol fractions were not run off in this case,

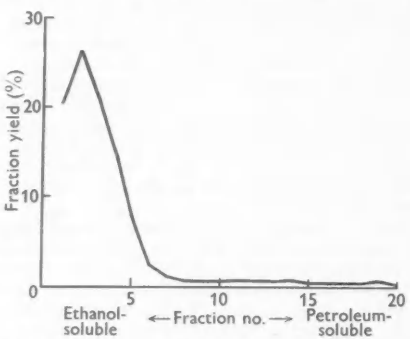

Fig. 4. Haddock flesh lipids. Counter-current distribution of acetone-soluble fraction *B′* between light petroleum and 85% (v/v) ethanol. Curve passes through experimental value for every fraction.

the total contents of each flask forming one fraction. The weight distribution is shown in Fig. 4. Fractions 1–6 formed a uniform material and were combined (30·1 g.). An attempt to resolve it further by distribution between light petroleum and tetrahydrofurfuryl alcohol was unsuccessful. Fractions 7–20 ranged in appearance from waxy solids to viscous gums, and were combined to give 2·4 g. of mainly non-phosphatidic material. The latter was too small for adequate examination, but on saponification it yielded 28% unsaponifiable matter and 36% fatty acids. The former appeared to be a mixture of aliphatic alcohols and other (possibly hydrocarbon) material; acetates: I_2 val., 91, sap. equiv., 660. This fraction also contained 1·0% choline, 0·04% serine + ethanolamine N, 0·26% glycerophosphate P, and 0·48% free phosphate P after alkaline hydrolysis. The fatty acids on lead salt/alcohol separation gave 42·5% solid acids (I_2 val., 39·4) and 57·5% liquid acids (I_2 val., 231·6).

The main phospholipin fraction (1–6), in spite of its relative impurity (choline, 9·4%; glycerophosphate P, 2·2%; acid hydrolysable P, 0·1%; other P, 0·6%) was treated as one substance. Saponification yielded 2% unsaponifiable matter and 58·5% fatty acids. The former was apparently higher aliphatic alcohols; acetates: I_2 val., 147, sap. equiv., 334. The fatty acids were analysed by distillation of the methyl esters, the results being given in Table 2.

The summation of constituent lipids in fraction *B′* would appear to be lecithin (from choline data) 60·6%, wax esters 5·8%, hydrocarbons, etc. 1·0%, unidentified 32·6%. The proportion of unidentified material is far higher than in fraction *A*. Most of it (28·9%) occurs in the main phospholipin fraction (1–6). It is not all non-lipid in nature since fractions 1–6 yielded 58·5% fatty acids, whereas lecithin and wax together would only yield 48·6% fatty acids. Since the glycerophosphate content is in close agreement with the choline content, this excess fatty acid is not present in phospholipins based on glycerophosphoric acid. In the absence of more information, it is impossible to say how much of the 32·6% unidentified material is non-lipid in nature. The small content of plasmalogens, about 3·6% if based on the classical glycerylphosphorylethanolamine, would form part of it.

DISCUSSION

The relative importance of the various lipids described here can be better appreciated by reference to the extraction data in the preceding paper (Lovern, 1953). The acetone-extracted lipids amounted to 0·57% of the fresh weight of the tissue and to almost half of the total tissue lipids. The material in the other extracts, although not yet examined in detail, is clearly more allied to the phospholipins and cerebrosides than to such substances as triglycerides, sterols, waxes, etc. Of the total acetone extract, all the triglyceride and cholesterol was concentrated into fraction *A* (35·7% of the total), of which in turn they comprised only the small percentages shown in Table 3. Assuming that all lipid fractions still to be examined are free from triglyceride and cholesterol, the total content of these in the haddock flesh is triglyceride 0·014%, cholesterol esters 0·020% and free cholesterol 0·036%. The position as regards wax esters is less clear, since it is a fairly common finding that unsaponifiable matter, presumably alcoholic, occurs in the hydrolytic products of crude phospholipin preparations, e.g. in those of ox brain (Lovern, 1952), ox, pig and sheep liver (Hilditch & Shorland, 1937) and soya bean and rapeseed

(Hilditch & Pedelty, 1937). Most workers with phospholipins have not looked for it. This unsaponifiable matter may not arise, as assumed in Table 3, from waxes, but from still more complex compounds akin to phospholipins. The material listed as 'hydrocarbons, etc.' is the least well characterized of the lipids itemized; it is merely unsaponifiable matter in excess of available fatty acid or deduced from unexpectedly high saponification equivalents of acetylated unsaponifiable matter. Certain fractions of unsaponifiable matter were pooled and freed from cholesterol and after acetylation had a saponification equivalent of 1150, far above the expected value of about 330. This product contained 85·5% C and 11·0% H, confirming, therefore, that it almost certainly contained some hydrocarbon. The occurrence of more free fatty acid than triglyceride in the tissue is remarkable, although once again the total concentration in the tissue is very low, being 0·036%. The test on absolutely fresh fish at sea gave a value of 0·034%.

The only comparable data known to the authors are those of Kaucher, Galbraith, Button & Williams (1943) for cod muscle, which they found to contain (calculated to wet weight) about 2·4% lipid, made up of 0·55% triglyceride, 0·03% cholesterol esters, 0·05% free cholesterol, 1·07% phospholipins and 0·66% cerebrosides. This tissue differs from haddock flesh, therefore, in containing far more triglyceride and phospholipin plus cerebroside, the amounts of free and esterified cholesterol being of the same order. Neither in the present work nor in that of Kaucher *et al.* was the tissue purely skeletal muscle, but included connective tissue, nerves, blood vessels, blood and lymph. While the preponderance of true muscle tissue may be overwhelming for the bulk of the lipid constituents, these other admixed tissues may contribute significantly to trace constituents such as sterols and triglycerides.

It is common practice in preparing phospholipin extracts of tissues first to dehydrate the tissue with acetone, which also removes unwanted lipids such as fat and cholesterol. Such acetone extracts, especially the first, aqueous, one, are commonly discarded. The present results show that, at least in certain cases, such a practice will result in loss of phospholipin, and probably selective loss. We observed that even the first, largely aqueous, acetone extract contained, in addition to much non-lipid matter, lipids amounting to about 0·06% of the tissue weight (i.e. 10% of the total acetone-extractable lipids), and having roughly the same composition (sterols, free fatty acids, phospholipins, etc.) as the main acetone-extracted lipids.

Counter-current distribution

Certain general features of the counter-current distribution require comment. Thus, Fig. 3 shows that the free cholesterol is distributed in a broad band, irregular on its advancing (ethanol-soluble) side. A single compound should give a symmetrical and narrower band and the observed effect is probably due to the marked effect of phospholipins on the solubility properties of other admixed lipids and, in the high concentrations used, on the properties of the solvent pair. Thus, the cholesterol is probably 'carried along' by the preceding main phospholipin fraction, 1–12.

Counter-current distribution cannot be effective for the separation of compounds which have partition ratios so high or low that the substances are sharply concentrated at one end of the system, as is true for lecithin in Figs. 3 and 4. In fact, such lecithin concentrates contained up to 20% of other lipids (choline, unsaponifiable matter and total fatty acid data), together with non-lipid impurities.

Both in the present and in other experiments we have noted that lipids are generally distributed so that more unsaturated derivatives are carried further by the polar solvent. This is true for free fatty acids, triglycerides and wax esters. The distribution of these over a great part of the system in the case of fraction *A* may be partly a genuine distribution and partly a trailing effect of the preceding phospholipins. It should be noted that uncharacterized nitrogen, presumably non-lipid, was similarly found all through the system.

'*Excess*' *nitrogen*

The nature of the 'excess' nitrogen is not clear. Part of it is liberated as ammonia during alkaline hydrolysis or addition of alkali to acid hydrolysates. This may well arise from urea, a known contaminant of some phospholipins, e.g. from blood (Christensen, 1939; Folch & Van Slyke, 1939). The balance of excess nitrogen is not present as an amino-acid, since a test with ninhydrin on a paper chromatogram showed only the expected traces of serine and ethanolamine. Glutamic acid, reported as a constituent of phospholipins (Hecht & Mink, 1952) was not present. Paper chromatography of the water-soluble products of alkaline hydrolysis of fraction 1–6 of *B'* showed the presence of a base reacting with phosphomolybdic acid but not with ninhydrin, also staining with iodine vapour, having an R_F value in butanol/acetic acid/water about half that of choline. It may correspond to a substance noted by Levine & Chargaff (1951). The lipid itself gave a strong Jaffé test for creatinine (equivalent, however, only to 0·04% creatinine) and this effect could not be removed by passing the lipid through a column of base-adsorbing ion-exchange resin. By contrast, the aqueous solution of an alkaline hydrolysate also gave a strong Jaffé reaction, the chromogen being readily removed on a column. While this test is not specific for creatinine, the latter is a known con-

stituent of fish flesh. If, in fact, creatinine occurs in these crude lipids, the evidence from adsorption experiments suggests that it is bound in some way to a larger molecule, possibly a lipid. Creatinine has been reported in a lipid extract of brain (Brante, 1949) and in MacLean & MacLean's (1927) 'carnithin'.

The nitrogen bound to the fatty acids has never been characterized. It would not appear to be present in such well-known derivatives as ureides since it cannot be removed by drastic hydrolysis. An association of nitrogen with partly oxidized fat is a well-known phenomenon in rancidity studies (Lea, 1938), but there is no evidence that similar effects are responsible in the case of phospholipins. This small amount of nitrogen was ignored in carrying out the fatty acid analyses.

Fatty acids of lecithins

The fatty acid compositions of the two lecithins (Table 2) exhibit several novel features, especially when compared with those of the depot fat. First, the absence of hexadecenoic acid, which is a common constituent of phospholipins from diverse species (Hilditch & Shorland, 1937; Shorland, 1951; Klenk & Böhm, 1951; Klenk, 1935) including phosphatides from fish liver and roe (Shorland & Hilditch, 1938; Shorland, 1939), and which is a characteristic universal constituent of fish depot fats. Secondly, the very high proportions of highly unsaturated C_{20} and C_{22} derivatives (60–70 % of the total acids) and the extremely high average unsaturation of these groups. Thirdly the low content of C_{18} unsaturated acids, normally a major constituent of phospholipins, coupled once again with unusually high average unsaturation. In fact, such a concentration of highly unsaturated derivatives has never previously been reported in phospholipin analysis. The fish liver and roe phosphatides mentioned above had fatty acid compositions closely similar to those of most fish depot fats. Anno (1949) reported that the lecithin fraction of salmon egg lipids contained oleic acid as the principal unsaturated acid, with only small amounts of highly unsaturated C_{20} and C_{22} acids, and Klenk (1933) found considerable proportions of highly unsaturated C_{20} and C_{22} acids in the liver phosphatides of a shark, although they were absent from the liver glycerides of this species.

Until we have examined the other phospholipins in our series of extracts it is impossible to say whether the exceptional fatty acid compositions reported here are a result of fractionation by acetone-solubility or are a general feature of fish-muscle phospholipins. The only previous comparison of the acetone-soluble and acetone-insoluble phosphatides of a tissue appears to be that of Hilditch & Shorland (1937) with sheep liver. The differences were not striking, but there was a tendency for higher unsaturation in the acetone-soluble fraction, due to increased proportions of highly unsaturated C_{22} acids, largely at the expense of less-highly unsaturated C_{18} acids.

In comparing these fatty acid analyses with those of other workers it should be remembered that the present fractions represented impure lecithins (up to 20 % other lipids) and that both, especially *A*, contained a certain proportion of acetone-insoluble lecithin. It may be that if we had succeeded in obtaining a pure preparation of the acetone-soluble lecithins of haddock flesh we should have found an even higher concentration of C_{20} and C_{22} highly unsaturated acids. It is, however, notable that these products still contained about 20 % of saturated acids, the minimum reported by various workers for phosphatides of diverse origin. Snider (1936) concluded that the fatty acids of muscle phospholipins of many species contain about 27 % saturated acids, in contrast to 40 % in liver phospholipin fatty acids. Sinclair's (1935) work on the effect of dietary fat emphasized the significance of the ratio of saturated to unsaturated acids in phosphatides. Most determinations of detailed fatty acid composition have been concerned with the total phosphatide fraction of a tissue. Consideration of the iodine values of some of the extracts described in the preceding paper (Lovern, 1953) suggests that the fatty acids of the total phospholipin fraction of haddock flesh would be less rich in highly unsaturated derivatives than the subfractions described here.

Free fatty acids

Finally, attention may be drawn to the composition of the free fatty acid fraction, which in its saturated acids resembles the two lecithins rather than the depot fat, but in its unsaturated acids occupies an intermediate position, for both proportions and average unsaturation of homologous groups. The significance of this fraction cannot be assessed until data are available on all the other lipids of the tissue.

SUMMARY

1. An acetone extract of haddock flesh was separated into three fractions comprising (*A*) acetone-soluble non-phosphatidic lipids plus some lecithin, (*B'*) mainly lecithin soluble in acetone in the absence of added electrolytes, and (*C*) acetone-insoluble lipids. The lecithin in (*A*) was partly genuinely soluble in acetone and partly soluble in the presence of other lipids. The significance of acetone-insolubility in the preparation of phospholipins is discussed.

2. Fractions *A* and *B′* were studied by countercurrent distribution between light petroleum and 85% (v/v) ethanol, followed by pooling of certain fractions on the basis of similar chemical compositions and analysis of the pooled material for various lipid constituents.

3. Fraction *A* consisted of about 32% lecithin, 18% free cholesterol, 18% free fatty acids, 10% cholesterol esters, 8% waxes, 7% triglycerides, 2% hydrocarbons, etc. and 5% non-lipid matter. The free fatty acids were a genuine constituent of haddock flesh and not a post-mortem artifact. Fraction *B′* consisted of about 61% lecithin, 6% waxes, 1% hydrocarbons, etc., and 32% unidentified material, including probably 4% plasmalogens.

4. Detailed fatty acid analyses of the lecithins in fractions *A* and *B′* revealed exceptionally high proportions of highly unsaturated C_{20} and C_{22} acids, with a curious total lack of hexadecenoic acid and unusually small amounts of C_{18} unsaturated acids. The free fatty acids were in some respects intermediate in type between those of the lecithins and those of haddock depot fat. The significance of these fatty acid compositions cannot be assessed until all the lipid fractions of the tissue have been similarly examined.

5. Certain fractions were quite rich in uncharacterized nitrogen, and early observations of the presence of firmly bound nitrogen in the fatty acids liberated from phospholipins were confirmed.

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The Lecithinase of *Clostridium bifermentans* Toxin

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Miles & Miles (1947, 1950) showed that culture filtrates of *Clostridium bifermentans*, an organism previously shown to give the Nagler reaction (Hayward, 1943), decomposed lecithin enzymically with production of a water-soluble organic phosphoric compound. This action was inhibited not only by homologous antisera but also, though less specifically, by *Clostridium welchii* antitoxin. Miles & Miles concluded from their experimental evidence that the action of the *Cl. bifermentans* filtrates was due to a lecithinase of the same biochemical type as the *Cl. welchii* lecithinase (*Cl. welchii* α-toxin), i.e. a lecithinase *C*, decomposing lecithin in a single step to phosphorylcholine and a diglyceride (Macfarlane & Knight, 1941). Miles & Miles also showed that, comparing the *Cl. welchii* and *Cl. bifermentans* preparations in enzymically equipotent doses, the *Cl. bifermentans* lecithinase was relatively non-toxic to mice, and relatively non-haemolytic towards red cells of various species.

The validity of any discussion of this interesting variation in the toxicity of similar enzymes depends on the identification of the *Cl. bifermentans* enzyme as a lecithinase *C*, and upon this point the evidence obtained by Miles & Miles is not conclusive. The chemical evidence adduced, i.e. the formation from lecithin of a water-soluble phosphorus compound, with liberation of choline on acid hydrolysis, does not exclude the presence of glycerylphosphorylcholine, nor establish that phosphorylcholine is a primary product. The discrepancies noted by Miles & Miles (1950) and also by Macfarlane (1948) between the capacities of the *Cl. welchii* and *Cl. bifermentans* preparations to decompose lecithin and the capacity to produce turbidity in egg yolk suggested that the *Cl. bifermentans* preparations might contain a lecithinase *B*, i.e. one producing fatty acids and glycerophosphorylcholine from lecithin, or even a mixture of lipolytic enzymes. The *Cl. bifermentans* enzyme also differed from the *Cl. welchii* enzyme in that calcium was apparently not required for activation.

Cl. bifermentans lecithinase is inhibited by *Cl. welchii* (antilecithinase) antitoxin. This fact, though it argues some similarity in structure, is not evidence that the enzymic groups are identical, for it is not known that the enzymic groups are antigenic *per se*. Indeed the fact that the antilecithinase to *Cl. oedematiens* β- or γ-lecithinases does not inhibit the heterologous enzyme indicates that the lecithinase group is not the only or the dominant antigenic group.

We have examined the biochemical action of the *Cl. bifermentans* preparations used by Miles & Miles in their studies, and have obtained more conclusive evidence that the lecithinase present is of the same biochemical type as *Cl. welchii* α-toxin.

MATERIALS AND METHODS

Enzyme preparations. Two freeze-dried preparations of *Cl. bifermentans* toxin, designated by us as B_1 and B_2, were used. These were derived from the least toxic strains used by Miles & Miles; for the same lecithinase activity, the toxicity was less than one-fortieth of that of *Cl. welchii* toxin.

The *Cl. welchii* toxin used was precipitated from a culture filtrate of *Cl. welchii*, S 107, with $(NH_4)_2SO_4$, dialysed and freeze-dried. A solution in 50% glycerol was used routinely.

Lecithin. This was prepared from egg yolk by the method of Macfarlane & Knight (1941).

Egg-yolk extract. Egg yolks were washed and beaten up in 0·9% NaCl to give a 10% (v/v) suspension. This was clarified by centrifugation for 30 min. at 7000 rev./min. in a Servall angle centrifuge. The preparation was stored at −10°, either alone, or buffered with 0·2 vol. of Palitsch borate buffer, pH 7·2, or 0·2 M-acetate buffer, pH 5·5.

Lecithinase activity. This was estimated in standard conditions: 1·0 ml. 2·5% (w/v) aqueous lecithin, 1·0 ml. Palitsch borate buffer, pH 7·2, containing 0·01 M-$CaCl_2$ and 2·0 ml. of toxin diluted in water were incubated 30 min. at 37°; 1·0 ml. 20% (w/v) trichloroacetic acid was added, the mixture filtered after 10 min. and the acid-soluble P estimated. The amount of toxin liberating 50 μg. P in these conditions was defined as an L unit. Within the range 0·5–3·0 L units the amount of P liberated was a linear function of the enzyme concentration.

Turbidity development in egg yolk. This was measured in an EEL photoelectric colorimeter. The toxin solution (1·0 ml.) was mixed with 1·0 ml. buffered egg-yolk extract and incubated at 37°. The turbidities were read against a blank containing egg yolk and water, and expressed as the scale reading. For quantities of toxin between 0·5 and 1·2 L units the turbidity was proportional to the concentration of enzyme, and within this range the turbidity was proportional to the time of incubation for at least 60 min.

Estimations of P were made colorimetrically (Martland & Robison, 1926) after wet-ashing.

RESULTS

Products of the hydrolysis of lecithin by Clostridium bifermentans *toxin*

Course of hydrolysis. An emulsion of lecithin (5 ml., 94 μmoles, 2·9 mg. P) in 0·002 M-$CaCl_2$, pH 7·2, was mixed with 60 mg. *Cl. bifermentans* toxin B_2 (30 L units) in 2 ml. water. Samples were removed for the initial analysis, and the remainder (87 μmoles) incubated at 37°. The hydrolysis was followed by titration of the acid formed with 0·05 N-NaOH; this was taken only to pH 7·2 (phenol red) to minimize saponification of glycerides. After 5·5 hr., 66 μmoles acid had been formed, and 77 μmoles acid-soluble P (89% hydrolysis). The mixture was then separated into water-soluble and ether-soluble fractions by three extractions with 10 ml. ether.

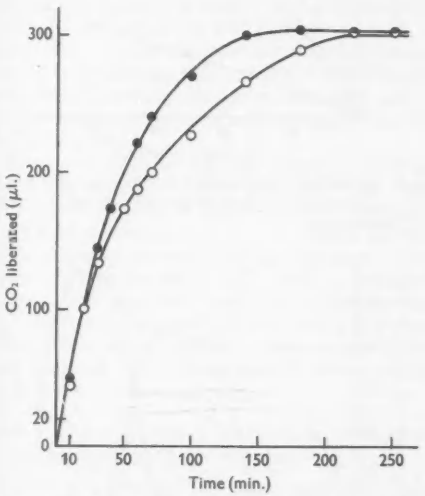

Fig. 1. Production of acid from lecithin by *Cl. welchii* and *Cl. bifermentans* toxins, followed manometrically. ○—○, 2 L units *Cl. bifermentans* B_2 toxin; ●—●, 2 L units *Cl. welchii* toxin.

Characterization of products. The water-soluble fraction after heating to 100° for 5 min. and filtration contained 1·9 mg. P, all in organic form (70% recovery of original phospholipin P). On treatment of a sample with a purified bone phosphomonoesterase at pH 8·0 in presence of 0·005 M-$MgCl_2$, all the P present was converted to orthophosphate in 1 hr. at 38°; a parallel test with potassium diphenyl phosphate showed that the bone phosphatase was free from diesterase. The hydrolysis product was therefore a monophosphoric ester.

The ether-soluble fraction gave 38·0 mg. of acetone-soluble material, acid value 2·9; calculated as oleylpalmitylglyceride, this represents 73% recovery on the original lecithin, and 83% on the actual hydrolysis. The fatty acids formed on saponification were not isolated.

The above hydrolysis was carried out on a small scale because of the low activity of the *Cl. bifermentans* enzyme and the limited amount available. As a check on the findings, a parallel experiment was run simultaneously with 30 L units of *Cl. welchii* toxin instead of *Cl. bifermentans* enzyme; the analytical figures and the recovery were practically identical. The production of acid from lecithin by equal quantities (L units) of the two lecithinases was also followed manometrically using the technique of Zamecnik, Brewster & Lipmann (1947) and found to correspond closely (Fig. 1). There appears little doubt that the *Cl. bifermentans* lecithinase is a lecithinase *C* liberating a monophosphoric ester, a neutral fat and one equivalent of acid from lecithin.

Tests for presence of other enzymes in Clostridium bifermentans *toxin*

Phosphodiesterase. The test mixture contained 2·0 ml. 0·1 M-citrate buffer, pH 6·5, 1·0 ml. 0·02 M-potassium diphenylphosphate, and 1·0 ml. toxin B_2 (10 mg.), with appropriate controls. After 30 min. at 37° the free phenol present was estimated using the Folin & Ciocalteu reagent. No liberation was observed, indicating that the toxin had no diesterase activity and that the phosphorylcholine formed during the hydrolysis of lecithin had not arisen by a secondary reaction.

Lipases. The action on triacetin, tributyrin and Tween 20 (a polyoxyalkylene derivative of sorbitan-monolaurate) was examined by the manometric technique of Singer & Hofstee (1948). With 50 mg. toxin B_2, in a total vol. of 3·3 ml., no increase in carbon dioxide evolution above that of the substrate without toxin took place in 1 hr. at 37°. The action of the toxin on triolein was examined by the method of Willstätter, Waldschmidt-Leitz & Memmen (1923), using 100 mg. toxin, with 1 hr. incubation at 37°, and again no lipase activity was detected.

Correlation between turbidity production and acid-soluble P *liberated in egg-yolk extract*

It appeared from the above experiments that the *Cl. bifermentans* toxin examined contained a lecithinase *C*, but no other lipolytic enzymes which could account for the discrepancies previously noted between the lecithinase activity and the rate of development of turbidity in egg yolk compared with *Cl. welchii* toxin. Nor does it appear that the discrepancy was due to proteolytic action (Miles & Miles, 1950). In the earlier experiments, however, the turbidity produced in egg yolk was referred to the number of L units used, i.e. to the activity of the two toxins acting on purified lecithin at pH 7·2. In the following experiments the turbidity produced was directly compared with the amount of acid-soluble P liberated in egg-yolk extract, as well as with the rate of hydrolysis of lecithin.

Dilutions of the *Cl. welchii* toxin and the *Cl. bifermentans* toxins B_1 and B_2 were made up so that 1 ml. samples produced turbidity at approximately the same rate when mixed with 1·0 ml. egg-yolk extract at pH 7·2 on incubation at 37°. The turbidity was measured at 20, 40 and 60 min. to check that it was proportional to the time of reaction, and at each time the acid-soluble P was estimated in 1·0 ml. samples: simultaneously, samples of the same toxin solutions were added to standard lecithinase test mixtures and the L units present determined in the usual way.

The results (Table 1) show that the ratio of turbidity to acid-soluble P production in egg yolk was practically the same for all three toxins, but the ratio of turbidity in egg yolk to acid-soluble P production from lecithin was considerably lower for

Table 1. *Correlation between turbidity production and hydrolysis of egg-yolk phospholipin by* Cl. bifermentans *and* Cl. welchii *lecithinases*

Reaction	*Cl. welchii*	*Cl. bifermentans* B_1	*Cl. bifermentans* B_2
(*a*) Turbidity in egg-yolk units/40 min.	46	46	54
(*b*) P liberation from egg yolk (μg./40 min.)	69	76	90
(*c*) P liberated from lecithin (μg./30 min.)	150	50	63
Ratio *a*/*b*	0·66	0·58	0·60
Ratio *a*/*c*	0·31	0·92	0·86

the *Cl. welchii* toxin than for the two *Cl. bifermentans* toxins, i.e. in the egg-yolk test the phospholipin was hydrolysed more slowly than in the standard lecithin test by *Cl. welchii* toxin, and more rapidly by the *Cl. bifermentans* toxins. Another experiment was made in which the rate of hydrolysis of egg-yolk phospholipin by the same number of L units of *Cl. welchii* and *Cl. bifermentans* toxin was compared. This experiment (Fig. 2) illustrates the difference in velocity and also shows that in these conditions both the toxins were apparently stable for 48 hr.

Effect of pH. Miles & Miles (1947) found the pH optimum for *Cl. bifermentans* lecithinase to lie between pH 5 and 6, whereas Macfarlane & Knight (1941) found that of the *Cl. welchii* was 7–7·6. The pH-activity curves for the samples of toxin used in the present experiments are shown in Fig. 3. The two curves intersect at pH 7·2 which was the initial pH used in the tests. It was found, however, that in the egg-yolk tests the pH fell rather rapidly to 6·0, and if a greater concentration of buffer were used this in itself inhibited the *Cl. welchii* enzyme (which was considerably purer than the *Cl. bifermentans* samples). Miles & Miles (1950) used a pH of 5·1 for their standard egg-yolk tests. It appears that the discrepancies between the lecithinase and the egg-yolk tests are largely due to the pH of the egg-yolk test being below the optimum for *Cl. welchii*

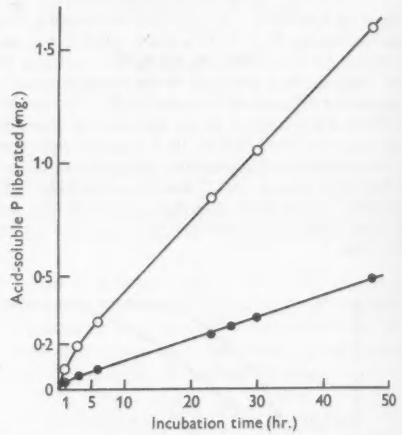

Fig. 2. Liberation of acid-soluble P from egg-yolk extract. ○—○, 2 L units *Cl. bifermentans* toxin; ●—●, 2 L units *Cl. welchii* toxin.

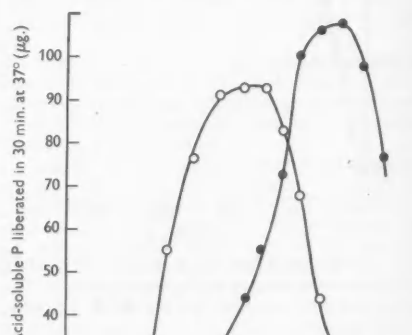

Fig. 3. pH-Activity curve of *Cl. welchii* and *Cl. bifermentans* lecithinases. ○—○, *Cl. bifermentans*; ●—●, *Cl. welchii*.

lecithinase, and to the difficulties of adjusting the salt concentrations so that both enzymes are equally affected. When the turbidity is referred to the hydrolysis of phospholipin in the egg-yolk

extract, rather than to the hydrolysis of lecithin in a separate test, there is no discrepancy, i.e. no evidence suggesting the existence of a second turbidity-producing factor.

Effect of fluoride and citrate on Clostridium bifermentans *lecithinase*

Miles & Miles (1947) found that *Cl. bifermentans* lecithinase differed from *Cl. welchii* lecithinase in that it was independent of activation by Ca^{2+}, i.e. the rate of hydrolysis was the same with and without Ca^{2+} and on addition of 0·01 M-oxalate. It has been found previously in this laboratory that when tests are made with toxins of low activity or with high concentrations of substrate it may be difficult to demonstrate activation by Ca^{2+}, as sufficient calcium may already be present in the toxin or substrate: in experiments with inhibitors such as fluoride and oxalate care must be taken that the pH and the time of contact are suitable for the effective precipitation of the insoluble calcium salt, e.g. at pH 5·1 the precipitation of calcium fluoride is slow and incomplete.

Effect of fluoride on egg-yolk reaction. Mixtures containing *Cl. bifermentans* toxin and egg yolk, pH 7·2, with or without sodium fluoride were used, and the tests arranged so that the sodium fluoride was in contact either with the egg yolk or the toxin for 15 min. before addition of the second compound, to compare with tests in which the sodium fluoride was added immediately after the two other components were mixed. Table 2 shows that inhibition by sodium fluoride in this system is demonstrable but it is by no means immediate.

Table 2. *Effect of fluoride on hydrolysis of egg-yolk phospholipin by* Cl. bifermentans *lecithinase*

(1·0 ml. *Cl. bifermentans* toxin, 1·0 ml. egg-yolk extract ±0·2 ml. 0·5 M-NaF in a total vol. 4·0 ml., incubated at 37°.)

System	P hydrolysed (μg.) 1 hr.	3 hr.	19 hr.
No fluoride	23	45	74
NaF added to toxin-yolk mixture	17	20	23
NaF added to yolk before incubation	17	17	21
NaF added to toxin before incubation	21	26	28

Effect of citrate on lecithinase reaction. The activity of *Cl. bifermentans* and *Cl. welchii* toxins in the standard lecithinase test at pH 7·2 in presence and absence of added Ca^{2+}, and in presence of citrate, was compared. The citrate was added to the lecithin 20 min. before the addition of the toxin. Table 3 shows that with both toxins the activity was increased in the presence of added Ca^{2+}, and considerably decreased by the prior addition of citrate.

Table 3. *Effect of citrate and* Ca^{2+} *on hydrolysis of lecithin by* Cl. bifermentans *and* Cl. welchii *toxins*

(1·0 ml. lecithin, 1·0 ml. borate buffer, pH 7·2, 1·0 ml. toxin, water or $CaCl_2$ to total vol. 4·0 ml., 30 min. at 37°.)

System	Acid-soluble P liberated (μg.) *Cl. welchii* toxin	*Cl. bifermentans* toxin
No added Ca^{2+}	75	143
0·01 M-$CaCl_2$	115	180
0·05 M-Citrate	20	30

DISCUSSION

The experiments described here confirm the conclusion reached by Miles & Miles (1947, 1950) that the lecithinase present in *Cl. bifermentans* culture filtrate is of the same biochemical type as that in *Cl. welchii* toxin (i.e. one splitting lecithin into phosphorylcholine and a diglyceride), but has a different pH optimum. It has been found that the action of this enzyme, like that of the *Cl. welchii* enzyme, is dependent on Ca^{2+} ions, and the lecithinase is apparently the only lipolytic enzyme present in the samples examined. It is desirable to establish this fact unequivocally, in view of the extraordinarily interesting differences in toxicity of the two enzymes shown by Miles & Miles (1950) and their possible use in comparative studies of enzyme-antienzyme systems.

Miles & Miles (1947) suggested that the difference in toxicity between the *Cl. bifermentans* and *Cl. welchii* enzymes might reside either in a readiness of absorption to certain tissue structures that is independent of lecithinase activity or in the presence in the body of activators to one but not to the other. There is, however, no *in vitro* evidence that any activation apart from the presence of Ca^{2+} ions is necessary. Later, Miles & Miles (1950) observed differences in the toxicity of lecithinase from different strains of *Cl. bifermentans*. Differences in the rate of attack of the lecithinases of *Cl. welchii* and *Cl. oedematiens* on the phospholipin of erythrocytes of various species are known. Macfarlane (1950) suggested that the differences in structure of the enzymes, which is clearly evidenced by their immunological specificity, conditioned the accessibility of the enzyme to its substrate when the substrate was itself part of a specific structure, and might also determine the toxicity of the enzymes and therefore the virulence of the microbes producing them.

SUMMARY

1. The lecithinase present in culture filtrates of *Clostridium bifermentans* is of the same biochemical type as *Clostridium welchii* lecithinase.

2. This enzyme, like the *Cl. welchii* lecithinase, is activated by Ca^{2+} ions.

We are greatly indebted to Mrs E. M. Miles for the gift of *Cl. bifermentans* toxins.

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Biochemical Studies of Toxic Agents

4. A STUDY OF ETHEREAL SULPHATE FORMATION *IN VIVO* USING RADIOACTIVE SULPHUR

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The present work was done to throw light on the question of whether administered inorganic sulphate can participate in the formation in the animal body of the so-called 'ethereal sulphates'. This matter has long been the subject of controversy. The problem is one which lends itself to investigation by means of tracer isotope techniques, and the present paper is an account of experiments in which ethereal sulphate formation *in vivo* was studied with the aid of radioactive sulphur (^{35}S).

In earlier papers of the present series descriptions have been given of the isolation of 2-amino-1-naphthylsulphuric acid from the urine of rats dosed with 2-naphthylamine (Manson & Young, 1950), and the isolation of 1-naphthylsulphuric acid and 2-naphthylsulphuric acid from the urine of rats dosed with 1- and 2-naphthol, respectively (Berenbom & Young, 1951). The formation of 2-amino-1-naphthylsulphuric acid from 2-naphthylamine is an example of ethereal sulphate production from a compound which, at least at the time of its administration, does not contain a phenolic group. On the other hand, the conversion of a naphthol to the corresponding naphthylsulphuric acid is typical of a reaction which is known to occur when many phenolic compounds are administered to animals. It appeared, therefore, that 2-naphthylamine and 1- or 2-naphthol would serve as suitable compounds in a study of the participation of administered inorganic sulphate in the biosynthesis of ethereal sulphates.

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Three groups of experiments were carried out. The first group consisted of determinations of the distribution of ^{35}S in the inorganic and total sulphate fractions of rat urine (and hence the ethereal sulphate fraction) following the administration of radioactive sodium sulphate alone, or simultaneously with the administration of 2-naphthylamine by another route. The results obtained indicated that the administered inorganic sulphate had participated in ethereal sulphate formation. This was confirmed in the second group of experiments. In one of these experiments radioactive 2-amino-1-naphthylsulphuric acid was isolated from the urine of rats dosed with 2-naphthylamine and radioactive sodium sulphate by different routes. In a similar experiment radioactive 2-naphthylsulphuric acid was isolated from the urine after the administration of 2-naphthol and radioactive sodium sulphate. In the third group of experiments it was shown that radioactive inorganic sulphate does not exchange in urine with the sulphate of the ethereal sulphate fraction.

EXPERIMENTAL AND RESULTS

Animals and dosing. The animals used were male white rats weighing between 170 and 210 g. During the experimental period they were housed in cages which permitted the collection of urine separate from the faeces. The rats were fed on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Ltd.) supplemented with fresh milk and whole wheat bread. In quantitative experiments the animals were allowed to feed twice daily for periods of 30 min. each from a metal cup designed to prevent scattering of food. In qualitative experiments the rats were fed in a separate cage twice daily for periods of 30 min. each and any

urine excreted during these periods was not collected. The animals had access to water at all times.

All dosing was carried out under ether anaesthesia. Solutions of sodium sulphate containing $Na_2{}^{35}SO_4$ were prepared using distilled water and were always administered by intraperitoneal injection. In one experiment, 2-naphthylamine suspended in 1% starch solution was administered by stomach tube. In other experiments 2-naphthylamine dissolved in olive oil or 2-naphthol dissolved in corn oil was injected subcutaneously in the lumbar region just lateral to the midline.

Determination of inorganic sulphate and total sulphate in urine. The method of Folin (1905) was used.

Measurement of ^{35}S *in the inorganic sulphate and total sulphate fractions of urine.* In order to measure the ^{35}S content of the inorganic sulphate fraction of urine, a small measured sample of urine in a 15 ml. centrifuge tube was made up to 6·0 ml. by the addition of distilled water. Two drops of a 0·04% solution of bromophenol blue in ethanol were then added, followed by N-HCl, drop by drop, until the colour of the solution was yellow without a trace of blue. 4·0 ml. of 95% ethanol were added and the sulphate was precipitated by the addition of 2·0 ml. of benzidine hydrochloride reagent (prepared by dissolving 5·0 g. of purified benzidine hydrochloride in 40 ml. of N-HCl and diluting the solution to 250 ml. with 50% (v/v) aqueous ethanol). The precipitate was stirred and after 30 min. it was separated by centrifuging. The supernatant liquid was discarded. The precipitate was washed in the centrifuge tube with four 5 ml. portions of 95% ethanol. After each washing the precipitate was separated by centrifuging and the supernatant liquid was discarded. The ^{35}S content of the precipitate was then measured in an ionization chamber with a direct current amplification system using an FP-54 tetrode valve (Young, Edson & McCarter, 1949).

In order to measure the ^{35}S content of the total sulphate fraction of the urine, 6·0 ml. of 4 N-HCl were added to a measured sample of urine (1–2 ml.) in a 50 ml. beaker, and the solution was evaporated to dryness on a water bath. The beaker was allowed to cool and its contents transferred to a 15 ml. centrifuge tube with addition of small portions of distilled water (total vol. 6·0 ml.) and 95% ethanol (total vol. 4·0 ml.). Benzidine sulphate was then precipitated, washed by the method already described, and its ^{35}S content measured by the method of Young *et al.* (1949).

The ^{35}S content of the ethereal sulphate fraction of the urine was calculated from the difference between the ^{35}S content of the inorganic sulphate fraction and that of the total sulphate fraction.

Measurement of ^{35}S *in organic compounds.* The method of Young *et al.* (1949) was used. The compound was oxidized by the Carius method and the H_2SO_4 formed in the process was separated as benzidine sulphate, the ^{35}S content of which was then measured.

Distribution of ^{35}S *in the inorganic, ethereal and total sulphate fractions of urine following the administration of radioactive* Na_2SO_4 *alone or with 2-naphthylamine*

Two rats (weight 182 and 185 g.) each received by intraperitoneal injection 0·5 ml. of a solution of radioactive Na_2SO_4 containing 0·1 mg. of sulphate S. The animals were placed in a metabolism cage and the urine was collected for the periods 0–12, 12–24, 24–28 and 48–72 hr. after dosing. At the end of 72 hr. each rat was again given the same amount of radioactive Na_2SO_4 by intraperitoneal injection, and in addition it received by stomach tube 0·050 g. of 2-naphthylamine in 1·0 ml. of 1% starch solution. The rats were returned to the metabolism cage and the urine was collected for the following 72 hr. at the same intervals as before. The urines were analysed for their contents of inorganic sulphate S (IS) and total sulphate S (TS), and from the data obtained the ethereal sulphate S (ES) content was calculated. The ^{35}S contents of the inorganic sulphate fraction ($I^{35}S$) and the total sulphate fraction ($T^{35}S$) were measured and from the results obtained the ^{35}S content of the ethereal sulphate fraction ($E^{35}S$) was calculated.

It should be noted that the amounts of Na_2SO_4 injected (0·1 mg. of sulphate S per rat) were less than 2% of the average daily excretion of TS in the urine of each rat, and the administration of such amounts of inorganic sulphate would not be expected to produce any appreciable disturbance of normal metabolism. The results obtained are shown in Table 1.

From the data in Table 1 it is seen that when radioactive Na_2SO_4 was given alone, 6% of the ^{35}S appeared as $E^{35}S$ in the 72 hr. period following dosing, and this indicates that a small amount of the administered inorganic sulphate was used in the synthesis of ethereal sulphates normally present in the urine. When 2-naphthylamine was administered together with the radioactive Na_2SO_4 there was a marked increase in ES. This was to be expected, for it is known that in the rat 2-naphthylamine is converted to 2-amino-1-naphthylsulphuric acid (Manson & Young, 1950). Under these conditions it was found that 48% of the ^{35}S given was excreted in the urine as $E^{35}S$ and this suggests that the administered inorganic sulphate participated in the formation of 2-amino-1-naphthylsulphuric acid. It is interesting to note that the rate at which the ^{35}S was excreted in the urine as $T^{35}S$ was not influenced significantly by the extent to which it took part in ethereal sulphate formation.

An experiment similar to that just described, except that the 2-naphthylamine was administered by subcutaneous injection, was carried out. Two rats were used and each received 0·4 ml. of radioactive Na_2SO_4 solution containing 0·07 mg. of sulphate S by intraperitoneal injection. After 72 hr. this dose was repeated and each rat was also given a subcutaneous injection of 0·025 g. of 2-naphthylamine in 1·0 ml. of olive oil. The results obtained were similar to those shown in Table 1. In the 72 hr. after radioactive Na_2SO_4 had been given alone, 5% of the ^{35}S was excreted in the ethereal sulphate fraction of the urine, whereas when radioactive Na_2SO_4 and 2-naphthylamine were given simultaneously, 44% of the ^{35}S was excreted in the ethereal sulphate fraction in the 72 hr. after dosing.

Table 1. *The excretion of inorganic, ethereal and total sulphate* S (IS, ES *and* TS) *and the distribution of* ^{35}S *in the inorganic, ethereal and total sulphate fractions of the urine* ($I^{35}S$, $E^{35}S$, $T^{35}S$) *of two rats following the intraperitoneal injection of radioactive* Na_2SO_4 (0·1 *mg. of sulphate* S *per rat*) *alone or with 2-naphthylamine* (0·050 *g. per rat*) *given by a stomach tube*

Period after dosing (hr.)	Sulphate S (mg.)			^{35}S (% of ^{35}S administered)		
	IS	ES	TS	$I^{35}S$	$E^{35}S$	$T^{35}S$
	Radioactive Na_2SO_4 administered					
0–12	7·4	1·2	8·6	73	4	77
12–24	6·6	0·8	7·4	10	1	11
24–48	12·6	1·6	14·2	6	1	7
48–72	8·6	1·6	10·2	—	—	—
0–72	35·2	5·2	40·4	89	6	95
	2-Naphthylamine and radioactive Na_2SO_4 administered					
0–12	1·6	3·4	5·0	39	38	77
12–24	0·6	5·0	5·6	1	9	10
24–48	8·2	2·4	10·6	4	1	5
48–72	8·4	1·6	10·2	—	—	—
0–72	18·8	12·4	31·2	44	48	92

Isolation of radioactive 2-amino-1-naphthylsulphuric acid from the urine of rats dosed with 2-naphthylamine and radioactive Na_2SO_4

Confirmation of the use of administered inorganic sulphate in the synthesis of 2-amino-1-naphthylsulphuric acid in rats dosed with 2-naphthylamine was obtained by the isolation of the radioactive sulphuric acid derivative from the urine of rats which had been dosed with 2-naphthylamine and radioactive Na_2SO_4.

Twenty-four rats were each given 0·025 g. of 2-naphthylamine in 0·5 ml. of olive oil by subcutaneous injection and at the same time each was given 0·5 ml. of radioactive Na_2SO_4 solution containing 0·12 mg. of sulphate S by intraperitoneal injection. The animals were dosed again 24 hr. later. They were housed in metabolism cages and the urine was collected daily for 3 days after the first dosing. As a measure of protection against the oxidation of urinary constituents, about 0·1 g. of sodium hydrosulphite was placed in each of the urine collection bottles at the start of each day's collection of urine. The collected urine was stored in the refrigerator. The total volume of urine obtained was 900 ml. Measured portions of the urine were analysed for $I^{35}S$ and $T^{35}S$ and the results were used to calculate the $E^{35}S$ content.

The results obtained, expressed as percentages of the ^{35}S administered, were as follows: $I^{35}S$, 43; $E^{35}S$, 34; $T^{35}S$, 77 %. The remainder of the urine was used for the isolation of 2-amino-1-naphthylsulphuric acid by a method which closely resembled that described by Manson & Young (1950).

The urine was first extracted for a total of 30 hr. in a continuous extractor with 500 ml. of peroxide-free ether to remove 2-naphthylamine and 2-acetamido-6-naphthol. The ether extract was discarded. The ether dissolved in the ether-extracted urine was removed under reduced pressure at room temperature, and the urine was then acidified to pH 2 by the addition of conc. HCl. It was then extracted with one 200 ml. portion and five 100 ml. portions of *n*-butanol saturated with water. The emulsions which formed during these extractions were broken by centrifuging. The butanol extracts were combined, made alkaline with ammonia and evaporated to dryness under reduced pressure at a temperature which did not exceed 45°. The residue was taken up in small portions (total vol. 60 ml.) of a mixture of 90 parts of ethanol and 10 parts of conc. HCl (v/v). This gave a purple-coloured solution which yielded a light-coloured precipitate after standing overnight in the refrigerator. The precipitate was filtered and washed with several 2 ml. portions of the acid-ethanol mixture. The residue was dried *in vacuo* and it was then dissolved in 15 ml. of N-KOH. The solution was filtered and acidified with conc. HCl. The precipitate which formed was filtered and washed with several 2 ml. portions of distilled water. A light-brown product was obtained which, after drying over P_2O_5 *in vacuo*, weighed 0·071 g. This material darkened at 216° and melted with decomposition at 221–222° (this and other melting points reported herein are uncorrected unless otherwise stated). Wiley (1938) reported a m.p. (corr.) of 224° for 2-amino-1-naphthylsulphuric acid isolated from the urine of dogs dosed with 2-naphthylamine. (Found: C, 49·9; H, 3·6; S, 13·1. Calc. for $C_{10}H_9O_4NS$: C, 50·2; H, 3·8; S, 13·4 %.)

A sample of the compound was analysed for its ^{35}S content and the remainder was dissolved in N-KOH in a centrifuge tube. The solution was acidified with conc. HCl and the precipitate was separated by centrifuging. The precipitate was washed with two 1 ml. portions of distilled water and after each washing it was separated by centrifuging. The compound was then dried over P_2O_5 *in vacuo* and samples were taken for the determination of the melting point and the measurement of the ^{35}S content.

The remainder of the compound was dissolved in 5 ml. of warm water and the solution was left overnight in the refrigerator. The precipitate which formed was separated by centrifuging and was washed in the centrifuge tube with two 1 ml. portions of distilled water. The product was dried over P_2O_5 *in vacuo* and its melting point and ^{35}S content were determined.

In the form in which its melting point was first determined the compound melted at 221–222°.

After being recrystallized it melted at 221–222°, and after further recrystallization the melting point was 222–223°. The corresponding ^{35}S contents of the compound (expressed as percentages of the administered ^{35}S per mg. of S in the compound) were 0·310, 0·310 and 0·307%, respectively. As the specific activity of the compound was not changed significantly by recrystallization, it is concluded that the compound isolated from the urine of rats dosed with 2-naphthylamine and radioactive sodium sulphate was radioactive 2-amino-1-naphthylsulphuric acid.

Isolation of radioactive 2-naphthylsulphuric acid from the urine of rats dosed with 2-naphthol and radioactive Na_2SO_4

In the experiment just described it is probable that 2-naphthylamine was first converted in the rat to 2-amino-1-naphthol, and that this compound was then converted to 2-amino-1-naphthylsulphuric acid. Elson, Goulden & Warren (1946) have suggested, however, that *p*-aminophenylsulphuric acid may be formed from aniline in the rat by a process which is independent of the formation of *p*-aminophenol. If this is so, the conversion of a non-phenolic carbocyclic compound, such as 2-naphthylamine, to an ethereal sulphate *in vivo*, might not involve the intermediate formation of a phenol. It was decided, therefore, to determine whether administered inorganic sulphate can participate in the rat in the formation of an ethereal sulphate from an administered phenolic compound. With this object in view, 2-naphthylsulphuric acid was isolated from the urine of rats dosed with 2-naphthol and radioactive Na_2SO_4. The 2-naphthylsulphuric acid was isolated in the form of its *p*-toluidine salt, and the method used was based on that described by Berenbom & Young (1951).

Each of twenty-four rats (weights 170–200 g.) received by subcutaneous injection 0·100 g. of 2-naphthol dissolved in 1·0 ml. of corn oil, and at the same time they were each given by intraperitoneal injection 0·6 ml. of an aqueous solution of radioactive Na_2SO_4 containing 0·1 mg. of sulphate S. The animals were dosed in this way once a day on three successive days. They were kept in metabolism cages, and the urine was collected for 88 hr. from the time of the first dosing and was stored in the refrigerator. The volume of the urine was 950 ml. Measured portions of the urine were analysed for $I^{35}S$ and $T^{35}S$, and the values obtained were used to calculate the content of $E^{35}S$.

The results obtained, expressed as percentages of the ^{35}S administered, were as follows: $I^{35}S$, 16; $E^{35}S$, 46; $T^{35}S$, 62%. The high proportion of the ^{35}S which appeared as $E^{35}S$ suggested that radioactive 2-naphthylsulphuric acid was present in the urine.

After the removal of samples for analysis, the remainder of the urine was adjusted to pH 8–9 by the addition of 20% (w/v) NaOH solution and it was then concentrated at 50–60° under reduced pressure to one-tenth of its original volume. The concentrated urine was transferred to a 250 ml. centrifuge bottle and was extracted with five 100 ml. portions of *n*-butanol. The urine was shaken with each portion of butanol for 15 min. on a mechanical shaker and the emulsions which formed were broken by centrifuging. After each extraction, 10 ml. of water were added to the urine to maintain its volume. The butanol extracts were combined and evaporated to dryness at 50–60° under reduced pressure.

The residue was dissolved in 40 ml. of water at 60–70° and 3·0 g. of *p*-toluidine hydrochloride were added with stirring. The solution was left in the refrigerator overnight and was then filtered. The precipitate (*P*-1) consisted mostly of glucuronide, but it gave a slight positive test for ethereal sulphate. The filtrate (*F*-1) gave a strong positive test for ethereal sulphate. The precipitate, *P*-1, was dissolved in a minimum of boiling water, charcoal was added, and the solution was filtered. The filtrate was left in the refrigerator and gave a precipitate (*P*-2) which consisted of glucuronide and gave no test for ethereal sulphate. The filtrate (*F*-2), which contained ethereal sulphate, was combined with *F*-1, and the combined solutions were evaporated to 40 ml. at 50–60° under reduced pressure. This concentrate gave a precipitate (*P*-3) after it had stood in the refrigerator. This precipitate gave no test for ethereal sulphate. The filtrate (*F*-3) was evaporated to dryness at 50–60° under reduced pressure.

The residue was extracted with 60 ml. of boiling ethanol and the extract was cooled. The precipitate (*P*-4) which formed consisted of NaCl and *p*-toluidine hydrochloride. The filtrate (*F*-4) was evaporated to dryness under reduced pressure at 50–60°, and the residue obtained was dissolved in a minimum of boiling water and treated with charcoal. The solution was filtered, and after standing in the refrigerator it yielded a precipitate (*P*-5) which gave a strongly positive test for ethereal sulphate and a weakly positive test for glucuronide. After being recrystallized twice from hot water this material was free from glucuronide. The product weighed 0·307 g.; m.p. 156–158° with previous darkening and subsequent decomposition. (Found: C, 61·7; H, 5·1; S, 9·5. Calc. for $C_{17}H_{17}O_4NS$: C, 61·6; H, 5·1; S, 9·7%.)

Potassium 2-naphthylsulphate was synthesized by the method of Feigenbaum & Neuberg (1941), and 1·0 g. of this compound when converted to *p*-toluidine 2-naphthylsulphate by the method of Berenbom & Young (1951) yielded 0·570 g. of purified product. (Found: C, 61·5; H, 5·0; S, 9·5. Calc. for $C_{17}H_{17}O_4NS$: C, 61·6; H, 5·1; S, 9·7%.) The synthetic compound melted at 156–158° with decomposition, and the melting point was unchanged when the compound was mixed with the product isolated from urine.

A sample of the *p*-toluidine 2-naphthylsulphate isolated from urine was analysed for its ^{35}S content. The remainder of the compound was recrystallized twice from water, and after each crystallization it was dried over P_2O_5 *in vacuo* and samples were taken for the determination of the melting point and the measurement of the ^{35}S content. The melting point of the compound was unchanged by recrystallization. The ^{35}S contents of the compound after

successive crystallizations (expressed as percentages of the administered ^{35}S per mg. S in the compound) were 0·216, 0·210 and 0·220 %. The variations from the mean value were within the limits of error of the analytical method used, and it is concluded that the compound isolated from the urine was radioactive *p*-toluidine 2-naphthylsulphate.

Investigation of the possible interchange of inorganic sulphate S and ethereal sulphate S in urine

The conclusion that administered inorganic sulphate can participate in the formation of ethereal sulphate *in vivo* would be invalidated if the sulphur of radioactive inorganic sulphate in the urine were to exchange with ethereal sulphate S. Experiments were therefore carried out to investigate this possibility for urine containing radioactive inorganic sulphate and 2-amino-1-naphthylsulphuric acid or 2-naphthylsulphuric acid.

On each of two successive days six rats were each given a subcutaneous injection of 0·025 g. of 2-naphthylamine dissolved in 0·5 ml. of olive oil. The urine was collected for 48 hr. after the first dosing. The volume of the urine was made up to 100 ml. and measured portions were analysed for IS and TS. An ES/TS value of 0·51, indicative of the presence of ethereal sulphate derived from 2-naphthylamine, was found. A solution (2 ml.) of radioactive Na_2SO_4 containing 2·6 mg. of sulphate S was added to the urine. The urine was allowed to stand at room temperature and after 24 and 96 hr. samples were taken and analysed for $I^{35}S$ and $T^{35}S$. The results obtained are shown in Table 2.

Another experiment was carried out in which each of six rats was injected subcutaneously with 0·100 g. of 2-naphthol dissolved in 1·0 ml. of corn oil. The urine was collected for 48 hr. after dosing. Analysis of the urine showed that its ES/TS value was high, namely 0·50. To the urine were added 5·0 ml. of a solution of radioactive Na_2SO_4 containing 3·3 mg. of sulphate S, and the volume was made to 100 ml. by the addition of water. The urine was allowed to stand at room temperature and samples taken after 24 and 72 hr. were analysed for $I^{35}S$ and $T^{35}S$. The results obtained are shown in Table 2.

Table 2. $I^{35}S$ *and* $T^{35}S$ *in urine at various times following the addition of radioactive* Na_2SO_4 *to the urine of rats dosed with 2-naphthylamine or 2-naphthol*

Time (hr.)	Urine from rats dosed with 2-naphthylamine		Urine from rats dosed with 2-naphthol	
	$I^{35}S$	$T^{35}S$	$I^{35}S$	$T^{35}S$
	Percentage of ^{35}S present at 0 hr.			
0	100	(100)	100	(100)
24	100	98	99	99
72	—	—	97	97
96	101	100	—	—

If ^{35}S had passed from the inorganic sulphate fraction of the urine to the ethereal sulphate fraction the $I^{35}S/T^{35}S$ value would have fallen. The results given in Table 2 show that this did not take place even though the experimental conditions (e.g. concentration of ethereal sulphate in the urine, duration of the experiments) were such that they gave ample opportunity for the occurrence of an exchange reaction. It is reasonable, therefore, to conclude that in the experiments described in the present paper, the appearance of ^{35}S in the ethereal sulphate fraction of the urine and the presence of ^{35}S in ethereal sulphates isolated from urine were not the result of exchange reactions taking place in the urine, but were the outcome of reactions occurring within the organism.

DISCUSSION

Baumann (1876), who was the first to show that formation of ethereal sulphates takes place in the body, was of the opinion that these compounds arise *in vivo* as a result of the union of sulphuric acid with a phenolic compound. Many years later, Sherwin and his co-workers (Rose, Shiple & Sherwin, 1924; Shiple, Muldoon & Sherwin, 1924) obtained results which led them to suggest that ethereal sulphates can arise by the oxidation of mercapturic acids in the body. No evidence of increased ethereal sulphate excretion after the administration of mercapturic acids was obtained, however, by Callow & Hele (1927), Coombs & Hele (1927) and Lawrie (1931). As a result of these and other studies on ethereal sulphate formation, Hele (1931) was led to conclude that in the organism ethereal sulphates are 'formed by the union of the sulphate ion with phenolic compounds'. More recently, work on the metabolism of phenols in tissue slices and homogenates (Bernheim & Bernheim, 1943; Storey, 1950; De Meio & Tkacz, 1952) has lent support to the view that inorganic sulphate is involved in ethereal sulphate formation *in vivo*.

If synthesis of ethereal sulphates takes place in the body in the manner envisaged by Baumann and by Hele, there arises the question of whether administered inorganic sulphate can take part in this process. The literature on this subject contains a number of conflicting observations. Whereas Baumann (1876) and Hele (1924, 1931) found that administered sodium sulphate could give rise to increased ethereal sulphate excretion by animals dosed with phenolic compounds, Tauber (1895), Rhode (1923), and Shiple *et al.* (1924) were unable to obtain evidence that this occurs.

As a result of the present work it is apparent that inorganic sulphate administered to rats by intraperitoneal injection can participate in ethereal sulphate synthesis. When sodium sulphate labelled with ^{35}S was administered alone, 5–6 % of the ^{35}S was excreted in the ethereal sulphate fraction of the urine. When an ethereal sulphate precursor,

2-naphthylamine, was given by stomach tube or injected subcutaneously and radioactive sodium sulphate was given by intraperitoneal injection at the same time, the excretion of ethereal sulphate was increased and 44–48 % of the administered ^{35}S appeared in the ethereal sulphate fraction of the urine. Furthermore, radioactive 2-amino-1-naphthylsulphuric acid and radioactive 2-naphthylsulphuric acid (as the *p*-toluidine salt) were isolated from the urine of rats which received radioactive sodium sulphate and were dosed with 2-naphthylamine and 2-naphthol, respectively. In our experiments the amount of radioactive sodium sulphate administered to each rat was less than 2 % of the normal daily excretion of total sulphate in the urine and the animals were maintained on a normal diet, whereas in a number of the balance experiments carried out by the earlier workers, large amounts of sulphate were administered and the protein intake of the animals was restricted.

Since the publication of the preliminary account of the present work (Laidlaw & Young, 1948), two papers bearing on the subject have appeared (Dziewiatkowski, 1949; Binkley, 1949).

Dziewiatkowski (1949) found that rats dosed by stomach tube or by intraperitoneal injection with sodium sulphate labelled with ^{35}S excreted some of the ^{35}S as ethereal sulphate sulphur in the urine. The amount of ^{35}S excreted in this form was greatly increased when phenol was administered, and this occurred when the phenol and radioactive sodium sulphate were both given by stomach tube and also when the phenol was given by this route while the radioactive sulphate was administered by intraperitoneal injection. These findings are similar to those reported by us.

Binkley (1949) described experiments in which he found that the addition of inorganic sulphate to the diet of rats receiving *p*-bromophenol neither produced a resumption of growth nor gave an increase in ethereal sulphate excretion. On the other hand, addition of cystine to the diet of these animals led to growth and an increased excretion of ethereal sulphate. Binkley considers that it is probable that no significant increase in the level of sulphate in the tissues is achieved by feeding sodium sulphate in the diet, whereas a single injection of sulphate, coincident with the administration of the phenol, would provide a more advantageous situation for conjugation. He is of the opinion that his findings and ours are not inconsistent if the mode of administration of the sulphate is taken into consideration.

While the extent to which administered inorganic sulphate takes part in ethereal sulphate formation in the animal body will clearly be influenced by a variety of factors, the experiments with radioactive sulphate have made it apparent that administered inorganic sulphate can participate in this process.

SUMMARY

1. Quantitative studies have been made of the excretion of radioactive sulphur (^{35}S) in the inorganic and total sulphate fractions of the urine of rats following the intraperitoneal injection of ^{35}S-labelled sodium sulphate alone, or simultaneously with the administration of 2-naphthylamine by stomach tube or by subcutaneous injection.

2. When radioactive sodium sulphate was given alone, 5–6 % of the ^{35}S administered was excreted in the ethereal sulphate fraction of the urine in the 72 hr. after dosing.

3. When 2-naphthylamine was administered as well as radioactive sulphate, the ethereal sulphate content of the urine was increased and 44–48 % of the ^{35}S administered was excreted in the ethereal sulphate fraction of the urine in the 72 hr. after dosing.

4. Radioactive 2-amino-1-naphthylsulphuric acid was isolated from the urine of rats injected intraperitoneally with ^{35}S-labelled sodium sulphate and given 2-naphthylamine by subcutaneous injection at the same time.

5. Radioactive 2-naphthylsulphuric acid (as the *p*-toluidine salt) was isolated from the urine of rats which were given ^{35}S-labelled radioactive sodium sulphate by intraperitoneal injection simultaneously with the subcutaneous injection of 2-naphthol.

6. It is concluded that intraperitoneally administered sodium sulphate can participate in the formation of ethereal sulphates in the rat.

An account of the work described in the present paper formed part of a thesis presented to the University of Toronto (Laidlaw, 1947), and a preliminary report of the investigation was presented to the Biochemical Society on 12 March 1948 (Laidlaw & Young, 1948).

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The Effect of Dinitro-*o*-cresol on the Deposition of Liver Glycogen in the Rat

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During the course of some experimental observations on the toxicity of dinitro-*o*-cresol (DNOC) a histological examination was made of the tissues of rats that had received a series of daily injections of a toxic dose (20 mg./kg.). The histological examination was essentially negative except for a somewhat unusual appearance of the liver cells. Special staining showed the liver to be packed with glycogen (Parker, Barnes & Denz, 1951). This was an unexpected observation because it is generally believed that the immediate effect of an injection of DNOC is to deplete the liver of its glycogen.

The effect of single and repeated doses of DNOC on the liver glycogen was, therefore, examined. The experiments showed that while the immediate effect of DNOC is to deplete the liver glycogen, the process is later reversed. The 24 hr. fasting levels of liver glycogen are always higher than normal in rats treated with one or a series of daily injections of DNOC.

In the course of the work an attempt was made to reduce the individual variation in the liver glycogen of normal fasting rats by submitting the animals to regular feeding regimes.

The normal practice in the animal house was to place in the cage a supply of rat cubes that was more than adequate for the needs of the rat during the next 24 hr. If food is suddenly withdrawn from these animals for a 24 hr. fast, the animals may differ in the amount of food each will have in its stomach at the time the food is withdrawn and the actual fast of some rats might be longer than 24 hr.

A regular feeding regime of two feeds a day, each of 1 hr. duration, and a single feed of 1 hr. was instituted. There was no reduction in the range of variation in individual animals, but the general fasting levels differed significantly.

These observations are discussed.

METHODS

Determination of liver glycogen, blood sugar and lactate. The liver glycogen was determined by the method of Good, Kramer & Somogyi (1933) on pieces of liver removed as quickly as possible from rats killed by guillotine. Although care was taken not to excite the animals immediately before death, an experiment was done that suggested that such a precaution was probably unnecessary. Two groups of four normal well-fed rats were killed. One group was kept quiet and killed, and the rats in the other group were excited just before being caught and killed. The glycogen levels in the two groups were $6{\cdot}92 \pm 2{\cdot}9$ and $7{\cdot}2 \pm 0{\cdot}72$% respectively. Blood sugar was determined by the method of Somogyi (1945) and blood lactate by that of Barker & Summerson (1941). All the results are expressed as means, together with the standard error.

Dinitro-o-cresol injections. DNOC purified by recrystallization from 50% ethanol was injected subcutaneously as a 1% (w/v) aqueous solution adjusted to pH 7. The dose was usually 20 mg./kg. which produces severe symptoms of poisoning. In order to reduce the number of deaths when the animals were to be given a series of injections, the cages were placed in a cool draughty corridor immediately after the animals had been injected.

Feeding regimes

Albino rats weighing 150–250 g. were used.

'*Continuous feeding.*' The rats were given each day a supply of cubes (Medical Research Council diet 41) more than adequate for their needs so that they had continuous access to food during the 24 hr.

Two meal regime. The rats were given cubes from 9–10 a.m. and 2–3 p.m. each day and had access to water at all times.

Single meal regime. The rats were given cubes only from 9 to 10 a.m. each day.

In the experiments with DNOC the rats were either on 'continuous feeding' or trained to eat a single feed for 1 hr. each day. In the latter case all rats were placed on the single feed regime for at least 3 weeks before they were used for experiments with DNOC to allow them to become accustomed to this new feeding regime.

RESULTS

Effect of feeding regime on fasting liver glycogen

The liver glycogen after a 24 hr. fast falls, but the extent of the fall depends upon the type of feeding regime to which the animals had been accustomed before being fasted.

Table 1. *The effect of the previous feeding regime on the liver glycogen of rats fasted for 24 and 48 hr.*

(*a*: rats with access to food throughout the 24 hr. *b*: rats accustomed to access to food for 1 hr. each day. Results expressed as means with standard errors.)

Feeding regime	Duration of fast (hr.)	No. of rats	Liver glycogen (%)
a	24	47	0·085±0·015
	48	6	0·26 ±0·07
b	24	53	0·70 ±0·07
	48	12	0·31 ±0·09

The results of a number of different observations have been collected and are presented in Table 1. They show that rats trained to eat their food during 1 hr. each day have a higher fasting level of liver glycogen than those accustomed to the continuous feeding regime. If the fast is continued for 48 hr. the liver glycogen levels of rats accustomed to continuous feeding rises again. In the rats trained to hourly feeds the level continues to fall, but not to levels as low as those seen in the continuously fed rats fasted for only 24 hr.

When rats were suddenly placed on the regular feeding regimes they took some time to adjust themselves to the new conditions and at first they lost weight, but by the end of 3 weeks they had regained this and were growing again.

Table 2. *Effect of different feeding regimes on the liver glycogen levels in normal fasting rats*

(All rats killed after 24 hr. fast. Each group contained 4 rats. Each group killed after 1, 2 and 3 weeks on the different feeding regimes. These regimes were (*a*) access to food for 1 hr. each 24 hr.; (*b*) access to food for two periods of 1 hr. each 24 hr.; and (*c*) access to food throughout the 24 hr. Results expressed as means with standard errors.)

Feeding regime	Liver glycogen (%)		
	1st week	2nd week	3rd week
a	1·04±0·20	0·48±0·20	0·49±0·04
b	0·27±0·10	0·22±0·06	0·04±0·01
c	0·03±0·004	0·07±0·005	0·03±0·004

An experiment was done in which rats were fasted for 24 hr. after periods of 1, 2 and 3 weeks on two regular feeding regimes and the liver glycogen estimated. The results are presented in Table 2 and indicate that it took at least 3 weeks for the animals to settle down to these new conditions as judged by the fasting level of their liver glycogen.

Rats were kept for several months on the 1 hr. feeding regime and remained perfectly healthy. At autopsy they were found to have greatly enlarged stomachs but no other abnormality.

Liver glycogen levels after DNOC

The original histological observations of the livers packed with glycogen were made on tissues from rats which had been on continuous feeding and were killed after a series of daily doses of DNOC. Table 3 gives amounts of glycogen found in the livers of rats on continuous feeding that had received a series of daily doses of DNOC compared with controls injected with saline. The rats were killed 24 hr. after the last injection of DNOC. The data confirm the histological observation that the non-fasting rat that has had previous injections of DNOC has more glycogen than normal in the liver. The immediate effect of a dose of DNOC is to reduce the amount of liver glycogen in the liver of the normal non-fasting rat (Table 4). It was, therefore, of interest to follow in more detail the changes that take place in the level of liver glycogen after injections of DNOC.

Table 3. *Effect of successive daily subcutaneous doses of DNOC on liver glycogen of rats*

(Daily dose of DNOC, 20 mg./kg. body wt. Rats were not fasted and were killed 24 hr. after the last injection of DNOC.)

No. of rats	No. of daily doses of DNOC	Liver glycogen (%)	
		With DNOC	Without DNOC
8	20	6·08±0·21	3·24±0·31
9	40	6·48±0·36	2·86±0·16

Table 4. *Effect of a single subcutaneous injection of DNOC on liver glycogen of rats*

(Dose of DNOC, 20 mg./kg. body weight. Rats were not fasted and were on the continuous feeding regime.)

Exp.	No. of rats	Time after DNOC (hr.)	Liver glycogen (%)	
			With DNOC	Without DNOC
1	3	0·5	0·78±0·28	3·45±0·16
2	4	1	0·73±0·12	1·94±0·29

It has been shown that the fasting liver glycogen is higher in rats accustomed to single feed regime than in those on continuous feeding. The effect of a series of injections of DNOC was to raise the fasting level in rats on either feeding regime. The animals were not given DNOC during the 24 hr. fasting period. The results from a number of experiments are collected and presented in Table 5. The

fasting liver glycogen was raised by a single injection and the effect was more marked after a series of 5–10 daily injections, but there was no significant increase in the fasting liver glycogen if the daily administration was carried on for longer periods which in some experiments extended to 40 days.

Table 5. *Effect of successive subcutaneous daily doses of DNOC on the liver glycogen of fasting rats*

(Rats received 10 daily doses of DNOC. 20 mg./kg. body weight. Rats fasted for 24 hr. after last dose of DNOC. The rats had been (*a*) on the continuous feeding regime, and (*b*) on the 1 hr. feeding regime.)

	Feeding regime	No. of rats	Liver glycogen (%)
a	With DNOC	17	0·59 ±0·06
	Without DNOC	47	0·085±0·015
b	With DNOC	33	3·34 ±0·31
	Without DNOC	53	0·70 ±0·07

Observations were then made during the whole 24 hr. period on rats accustomed to a single meal regime. After 3 weeks on this regime some of the rats were given DNOC (20 mg./kg.) immediately after the feeding period for 10 successive days. Groups of rats were killed at different times after the final dose of DNOC. The results are given in Table 6. They show that in the animals that had

Table 6. *Effect of successive daily subcutaneous doses of DNOC in liver glycogen of rats during 24 hr. period following the last dose of DNOC*

(All rats on 1 hr. feeding regime. DNOC, 20 mg./kg. body weight given immediately after hourly feed. All treated rats given ten doses of DNOC.)

Time after DNOC (hr.)		No. of rats	Liver glycogen (%)
2	With DNOC	10	0·48±0·16
	Without DNOC	15	3·96±0·23
4	With DNOC	9	3·48±0·83
	Without DNOC	10	4·97±0·45
8	With DNOC	19	4·62±0·59
	Without DNOC	18	7·16±0·54
12	With DNOC	6	4·11±0·72
	Without DNOC	8	8·82±0·38
24	With DNOC	20	4·64±0·43
	Without DNOC	20	0·84±0·25

received DNOC the liver glycogen was much lower than in the control rats 2 hr. after the meal, but later it rose. It did not, however, exceed the level in the normal rats until sometime after 12 hr. but by the end of 24 hr. the differences were well marked. Since the rats receiving DNOC must have started their last meal with a liver glycogen level of between 2 and 4 %, it is probable that the low level 2 hr. after the meal (0·48 %) must have resulted from an increased loss of glycogen from the liver and not from a failure to lay down more from products of digestion.

In other experiments the rats were killed at intervals between 24 and 48 hr. after the last dose of DNOC, having had a feed at the end of the first 24 hr. (Table 7). In these animals there was no fall in liver glycogen after the meal because no following DNOC had been given, but there was a greater quantity in the liver at 32 and 48 hr. in the DNOC-treated rats.

Table 7. *Effect of successive daily subcutaneous doses of DNOC on liver glycogen of rats during the period 24–48 hr. after the last doses of DNOC*

(Rats on 1 hr. feeding regime and given ten successive daily injections of DNOC (20 mg./kg.). Fed 24 hr. after the last dose of DNOC and killed at intervals thereafter.)

Time after DNOC (hr.)		No. of rats	Liver glycogen (%)
25	With DNOC	4	3·15±0·14
	Without DNOC	4	2·02±0·20
28	With DNOC	7	6·37±0·46
	Without DNOC	8	4·55±0·40
32	With DNOC	8	9·09±0·13
	Without DNOC	8	5·97±0·28
48	With DNOC	8	2·94±0·37
	Without DNOC	8	0·41±0·13

In one experiment the animals were killed 4, 8 and 24 hr. after their daily feed 7 days after receiving their last dose of DNOC. There was no difference in the glycogen levels in the control rats and those that had previously received DNOC.

Table 8. *Effect of a single injection of DNOC on the synthesis of liver glycogen of rats following glucose by mouth*

(Thirty-six rats fasted 24 hr. and then given 5 ml. 20% (w/v) glucose by mouth and divided into two groups of 18. One group were controls and the other injected subcutaneously with DNOC 20 mg./kg. body weight. These two groups were further subdivided and nine rats killed 2 and 4 hr. after receiving glucose.)

Time after glucose (hr.)	Liver glycogen (%)	
	With DNOC	Without DNOC
2	0·69±0·21	1·54±0·13
4	1·75±0·17	1·49±0·13

DNOC and glycogen synthesis in the liver of the fasting rat

The effect of DNOC on the synthesis of liver glycogen was examined in fasting rats that had been given glucose by mouth. DNOC was given immediately after the glucose and these rats, together with the controls given glucose and injections of saline, were killed 2 and 4 hr. later. The figures given in Table 8 show that there was no difference at

4 hr. in the amount of glycogen in the livers of the normal and poisoned rats. At 2 hr. there was still fluid in the stomachs of the DNOC rats while the stomachs of the normal rats were empty. Thus the lower level of glycogen in the liver at this time might partly be the result of poor absorption during the acute phase of poisoning.

DNOC and blood lactate

Cori & Cori (1928) found that after an injection of adrenaline the liver glycogen of rats fell rapidly but later rose above the starting level. They found a rise in the blood lactic acid in rats soon after the adrenaline had been given and suggested that lactic acid had been liberated from the muscles under the influence of the adrenaline, and that it was assimilated by the liver and converted into glycogen.

The blood sugar, blood lactic acid and liver glycogen were determined in rats killed at intervals after an injection of DNOC (20 mg./kg.). Although there was twice as much glycogen in the liver of those rats killed at 24 hr. that had received DNOC as in the controls, there had been no increase in the blood lactic acid levels of rats killed at 2, 4, 8, 12 and 16 hr. after the DNOC (Table 9).

Role of adrenals in the response to DNOC

The work of others (see below) has indicated that the rise in liver glycogen due to anoxia, alloxan diabetes and dietary changes may be mediated by the adrenals. Attempts to detect the effects of DNOC on the deposition of liver glycogen in adrenalectomized rats were made difficult by the greater susceptibility of the adrenalectomized rats to the toxic action of DNOC. While most adrenalectomized rats could withstand one injection of 20 mg./kg., a second injection on the following day killed the majority of these animals. In two experiments on male rats the adrenalectomized rats were given 20 mg./kg. DNOC on the first day and 15 mg./kg. on the second day. There was a 20 % mortality but the survivors were killed 24 hr. later, not having been fasted. In both experiments the group that had received DNOC had a higher liver glycogen than the untreated adrenalectomized rats but in only one experiment was the difference significant.

The effect of a series of daily injections of DNOC on the weight of the adrenals was examined. Increases in adrenal weight have been reported in rats with alloxan diabetes where there is also an increase in the liver glycogen levels (Bennett & Koneff, 1946). Rats were given a series of twenty and forty daily injections of DNOC (20 mg./kg.) and at the end of the period they were killed and the adrenals removed, cleaned free of fat and weighed. Control rats were given daily injections of saline. The results are given in Table 10 and show that there was a significant increase in the weights of the adrenals of male rats receiving DNOC but not of the females.

Table 9. *The effect of a subcutaneous injection of DNOC (20 mg./kg. body weight) upon the levels of liver glycogen, blood sugar and blood lactic acid of rats*

(All animals on 1 hr. feeding regime and groups of four killed at intervals after feeding. Control rats had received no DNOC.)

Time after feed (hr.)	With DNOC			Without DNOC		
	Glycogen (%)	Blood sugar (mg./100 ml.)	Lactic acid (mg./100 ml.)	Glycogen (%)	Blood sugar (mg./100 ml.)	Lactic acid (mg./100 ml.)
2	0·63±0·21	166±19	22	4·95±0·46	106±2·8	29
4	2·23±0·29	91±10	22	5·88±0·62	48±3·5	22
8	3·77±0·47	118±3	21	9·49±1·25	99±5·5	22
12	5·29±0·67	123±3	24	8·85±0·45	94±2·5	23
16	4·31±0·85	109±3	21	6·38±1·10	96±7·0	20
24	5·56±0·48	76±1·5	25	2·74±0·60	70±5·5	21

Table 10. *The effect of repeated daily injections of DNOC on the adrenal weights of rats*

(Groups of male and female rats given 20 or 40 daily subcutaneous injections of 20 mg. DNOC/kg. and killed. Adrenal weights expressed as mg./100 g. body wt.)

Sex	Treated with DNOC		Controls		t	P
	No.	Wt. of adrenal (mg.)	No.	Wt. of adrenal (mg.)		
M.	22	22·46±0·73	24	19·72±0·59	3·02	<0·01*
F.	9	35·28±1·64	8	32·56±1·24	1·24	<0·37†

* Difference highly significant.
† Difference not significant.
t, ratio of an observed deviation to its estimated standard deviation.
P, the probability of obtaining a random sample more divergent than the group observed (Fisher, 1948).

DISCUSSION

An explanation of the effects of a change in the feeding habits on the fasting liver glycogen levels is suggested by the work of Tepperman, Brobeck & Long (1942–3). They studied the metabolic changes in rats submitted to hypophysectomy and they found marked changes in the respiratory quotient during the 24 hr. In considering the causes of these changes they realized that hypophysectomy rendered the rats hyperphagic so that they ate all the food as soon as it was presented to them. When normal rats were trained to eat all their food during 1 hr. a day, they found changes in the R.Q. similar to those in hyperphagic hypophysectomized rats. Werthessen (1937) had also found violent fluctuations in the R.Q. of rats fed only once daily.

Tepperman *et al.* (1942–3) concluded that in the normal animal there were three reserves of energy—the liver glycogen, the body fat and the food in the gut. Normally, a rat has food in its gut for the whole 24 hr., but in rats trained to eat all their food during 1 hr. a day the gut is empty for about 12 hr. These rats will be accustomed to a 12 hr. fast every day and so may be better adjusted to a 24 hr. fast than those used to feeding at will during the 24 hr.

These observations suggest that care may be necessary in assessing the effects of drugs, hormones or dietary changes upon the fasting liver glycogen levels. Before conclusions can be drawn about the significance of any differences that may be observed, it will be necessary to be certain that any endocrine imbalance, change in the palatability of the diet or effect of a drug has not modified the feeding habits of the treated animals.

The effect of DNOC was studied in rats accustomed either to continuous feeding or to a single daily feed and was similar in both groups. One injection of DNOC (20 mg./kg.) into a rat produces a profound toxic reaction lasting a few hours (Parker *et al.* 1951). These rats always have a higher liver glycogen level than control untreated rats when examined 24 hr. after an injection of DNOC. This difference is observed after single or repeated daily doses of DNOC in rats fasted or fed during the 24 hr. period after the DNOC.

On the basis of the observations of Tepperman *et al.* (1942–3) on rats trained to a single daily meal, it is possible that the higher liver glycogen might represent the response of the animal to the fact that its intestine is empty for 12 hr. each day, which might constitute a loss of an important reserve of energy and create a need to maintain a higher liver glycogen reserve.

Mackay & Drury (1941) have shown that the amount of energy reserves laid down as glycogen in 24 hr. is only one-tenth of the total consumed as food during the same period. In the fasting animal it is the fat and in the fed animal the fat or intestinal contents that act as the main reserves of energy. Although the dose of DNOC may have some effect on the absorption of food from the gut, this effect was short-lived, for the fasted rat given glucose and then DNOC (20 mg./kg.) had laid down as much glycogen 4 hr. later as the untreated control (Table 8). It seems improbable, therefore, that the raised level of liver glycogen 24 hr. after a dose of DNOC can be attributed to an effect of the drug upon the absorption of food from the intestine with the imposition of an apparent fast upon the animal.

In recent years the influence of the adrenal upon the liver glycogen level has become well recognized. The effect that a number of entirely different procedures has upon the liver glycogen level has been attributed to an action on the adrenals. Mirski, Rosenbaum, Stein & Wertheimer (1938) found that the fasting level of glycogen was higher in rats receiving a diet of 70% protein than in those receiving a diet of 70% carbohydrate, a difference not seen in adrenalectomized rats. Evans (1934) found that a period of hypoxia increased the liver glycogen level in rats. This was not simply a reflexion of a slower rate of metabolism, because if the glycogen level was reduced by fasting at a normal atmospheric oxygen tension, it could then be raised by exposing the rats to a reduced oxygen tension. This effect is not seen in rats deprived of their adrenal cortex. Lewis, Thorn, Koepf & Dorrance (1942) showed that the effect of hypoxia on the liver glycogen was prevented by giving the animals 5% carbon dioxide. Heilman (1944) found that the addition of carbon dioxide prevented the hypertrophy of the adrenal cortex that normally occurs after periods of hypoxia. He suggests that the addition of carbon dioxide prevents the acid-base shift that will result from the hyperpnoea which occurs at low oxygen tensions, and that a disturbance of the mineral metabolism causes a response in the adrenal cortex which incidentally results in changes in the liver glycogen level. These observations have been confirmed by Langley, Nims & Clarke (1950).

In alloxan diabetes Tuerkischer & Wertheimer (1946) and Weber (1946) reported that the rat has a liver glycogen level higher than normal. Weber (1946) found that this did not take place in adrenalectomized rats. Bennett & Koneff (1946) found that there was an increase in the weights of the adrenals in rats rendered diabetic with alloxan. Morita & Orten (1950) reported that there was no correlation between the height of the blood sugar and the amount of glycogen in the liver of diabetic rats.

In the experiments with DNOC it has proved impossible to establish with certainty whether the adrenals play a part in bringing about the changes in liver glycogen levels after DNOC. Although the

weight of the glands in male rats was increased by repeated daily injections of DNOC the increase was not significant in the case of the female rats. It proved impossible to give adrenalectomized rats repeated daily injections of DNOC in doses that in normal rats would have been adequate to produce the late rise in the liver glycogen.

A third possibility was that the rise in liver glycogen after the immediate toxic effects of DNOC had disappeared, might be explained on lines similar to those suggested by Cori & Cori (1928) to explain the late rise in liver glycogen after injecting adrenaline into rats. Loomis & Lipmann (1948) first showed that dinitrophenol inhibited the uptake of phosphate by respiring mitochondrial preparations. Judah & Williams-Ashman (1951) and Judah (1951) have extended these observations and shown that under the influence of dinitrophenol or DNOC the uptake of inorganic phosphate progressively decreases but the consumption of oxygen is increased. The increased respiration rate of animals poisoned with DNOC indicates a greater oxygen consumption and presumably increasing amounts of substrate are sacrified to supply the energy needed for the increasingly inefficient phosphorylating processes. The sharp fall in the liver glycogen may be the reflexion of this demand for sources of energy.

When the acute phase of poisoning is over and the phosphorylating processes can again be carried out normally, there may well be an excess of metabolites remaining from the preceding period of increased oxidative activity. These may be taken up by the liver and converted into glycogen. Cori & Cori (1928) found a rise in the blood lactic acid after adrenaline, but no rise in blood lactic acid occurred after DNOC. This does not invalidate the hypothesis, but a search has not been made for changes in the blood levels of other metabolites.

It is, of course, possible that any changes in the blood level might be too small to detect. This might be due to their slow release or to ability of the liver to remove them so efficiently that no accumulation could take place in the blood, but only an accumulation of the glycogen formed from them in the liver.

SUMMARY

1. The effects of single and repeated toxic doses of dinitro-*o*-cresol (DNOC) upon the liver glycogen of the rat are described.

2. An initial fall in liver glycogen is followed by a progressive rise, so that after 24 hr. the level is higher in control animals.

3. A single dose of DNOC did not impair the absorption of glucose or laying down of glycogen in fasting rats.

4. Blood lactic acid levels are not affected by the doses of DNOC given.

5. Repeated doses of DNOC increased the weight of the male adrenal but not that of the female rat.

6. The findings are discussed and the changes in liver glycogen are taken to be a reflexion of the general metabolic disturbance produced by DNOC.

7. The level of liver glycogen in a normal rat fasted for 24 hr. depends upon the feeding habits to which the animals were accustomed before the fast.

I wish to thank Messrs J. A. E. Jarvis, B. W. Street and R. Manston for carrying out the numerous determinations recorded in this paper.

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The Effect of Carbonic Anhydrase on the Action of Yeast Carboxylase

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In the decarboxylation of pyruvic acid by yeast, the question of an initial formation of carbonic acid or of carbon dioxide may be investigated by the use of carbonic anhydrase. The use of this enzyme in such investigations was first suggested by Roughton and co-workers (Meldrum & Roughton, 1933; Ferguson & Roughton, 1934; Roughton, 1935). Roughton (1935) published a diagram (Krebs & Roughton, 1934) showing the effect of the enzyme on the carbon dioxide pressure when urease acts on urea.

The relation of ferment to substrate was such that the total urea-splitting occurred in a very short time. The liberation of free carbon dioxide as a primary product was shown by the rapid rise of the pressure in the manometer, followed by its slow decline (over about 10–15 min.) as carbon dioxide was taken up as HCO_3^- by the buffered medium. In the presence of a sufficient concentration of carbonic anhydrase no such initial fling and return of the carbon dioxide pressure occurs.

In repeating such experiments with carboxylase the difficulty lay in obtaining a preparation strong enough to act on the pyruvate substrate with adequate rapidity. Recently, Krebs & Roughton (1948) have so far fulfilled this requirement as to obtain results showing that, for the conditions studied, carbon dioxide is mainly the primary product.

In the investigation of the carboxylase action with carbonic anhydrase we used a somewhat different approach, namely strong concentrations of pyruvate, so as to produce a linear or near-linear rise of pressure over the time studied (of the order of 5 min.). Initial experiments (Conway & MacDonnell, 1945) seemed to indicate an increased carbon dioxide tension in the presence of carbonic anhydrase. These preliminary experiments were technically unsatisfactory. Also, the theoretical change of pressure arising from carbonic anhydrase would be very slight at the initial pH of 5·5. Owing, however, to the inadequate buffering this pH very rapidly changed to higher values, which appears to account for the differences found.

Subsequent experiments, better controlled (see O'Malley, 1947), supported the original conclusions and an account was delayed pending further clarification. Simultaneously, Krebs (1948) and Conway & O'Malley (1948) read communications concerning the effect of carbonic anhydrase on decarboxylation. Krebs advanced definite proof that, under the conditions studied, carbon dioxide was a primary product (though, as judged from the evidence here given, it was not necessarily the sole primary product of the carbon dioxide system). Our results supported the conclusion that whether carbon dioxide or carbonic acid was the main primary product depended on the conditions, and we gave equations, the derivations of which are described in this paper, for the theoretical evaluation of the effect of carbonic anhydrase, assuming a linear or near-linear rate of decarboxylation, as with strong pyruvate substrate. More recently, after a study of the best theoretical conditions, using the strong pyruvate substrate, a type of experiment was designed which would appear to furnish clear-cut results, the pressure changes being compared with the theoretical figure. This type of experiment only will be described in the present account.

THEORETICAL

If yeast carboxylase acts in a small volume of concentrated pyruvate solution, well buffered at pH 7·0, and CO_2 is liberated into a gaseous phase of constant volume, with adequate shaking and at constant temperature, equations may be deduced, described below, relating the CO_2 in the gaseous phase (and hence the change in pressure) with the time of action. It is assumed that the rate of decarboxylation is constant over the short period studied.

Symbols

n_1, n_2 = the number of moles of free CO_2 in the gaseous and liquid phases, respectively; n_3, n_4 = the number of moles of HCO_3^- and of H_2CO_3 in the liquid phase, respectively; V_1, V_2 = the volume, in litres, of the gaseous and liquid phases (V_2 throughout is $3{\cdot}4 \times 10^{-3}$ l., and $V_1 = 4{\cdot}6 \times 10^{-3}$ l. in most of the experiments); k_1, k_2 = the velocity constants of the reactions:

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad \text{and} \quad H_2CO_3 \rightarrow CO_2 + H_2O,$$

respectively (k_1 and $k_2 = 1{\cdot}5$ and 720 min.$^{-1}$ at 18° from the data collected by Roughton (1935)); a_{H^+} = the hydrogen-ion activity in the liquid phase,

and corresponds in the experiments to pH 7·0, as determined by the glass electrode;

$$K' = a_{H+} \times n_2/n_4 = 10^{-3\cdot5}, \text{ approx.};$$

$\lambda = \frac{n_2}{V_2} \Big/ \frac{n_1}{V_1}$, or the Ostwald absorption coefficient (= 0·7 approx.) as deduced from a consideration of the fluid composition in the decarboxylation studies described and various solubility data for CO_2 in the *International Critical Tables*; $S = Rt$, where S is the sum of the moles of the carbonic acid system, R a constant and t the time in minutes.

In addition, the following symbols have been used for convenience:

$$m = V_2\lambda/(V_2\lambda + V_1);$$
$$k_3 = k_2/(1 + K'/a_{H+});$$
$$\beta = k_3 + k_1 m.$$

From the above data the value of m is 0·34 and of k_3 and β 0·23 and 0·74 min.$^{-1}$, respectively.

Deduction of the equations relating n_1 to the time t

With H_2CO_3 as the primary product of decarboxylation, the rate of total free CO_2 production may be written

$$\frac{dn_1}{dt} + \frac{dn_2}{dt} = k_2 \frac{n_4}{V_2} V_2 - k_1 \frac{n_2}{V_2} V_2$$
$$= k_2 n_4 - k_1 n_2, \quad (1)$$

where CO_2 is the primary product,

$$\frac{dn_1}{dt} + \frac{dn_2}{dt} = k_2 n_4 - k_1 n_2 + \frac{dS}{dt}$$
$$= k_2 n_4 - k_1 n_2 + R, \quad (2)$$

Proceeding from equation (1),

$$\frac{dn_1}{dt} \times \frac{V_2\lambda}{V_1 m} = k_2 n_4 - k_1 n_1 \frac{V_2\lambda}{V_1}. \quad (3)$$

n_4 can also be expressed in terms of n_1 from

$$S = Rt = n_1 + n_2 + n_3 + n_4, \quad (4)$$
$$= n_1 \frac{V_2\lambda}{V_2 m} + n_4 \frac{k_2}{k_3}. \quad (5)$$

Inserting the value of n_4 from equation (5) in equation (3) one obtains

$$\frac{dn_1}{dt} + \beta n_1 = \frac{V_1 m R k_3}{V_2\lambda} \times t, \quad (6)$$

and proceeding from equations (2) and (5) in a similar way there follows

$$\frac{dn_1}{dt} + \beta n_1 = \left(\frac{V_1 m R k_3}{V_2\lambda} \times t\right) + \frac{R V_1 m}{V_2\lambda}. \quad (7)$$

With respect to the variables n_1 and t, equations (6) and (7) are linear differential equations of standard form. Integrating between zero time and time t with $n_1 = 0$ when $t = 0$, and with H_2CO_3 the primary product, there follows

$$\frac{n_1}{R} = \frac{m k_3 V_1}{\beta V_2 \lambda} \left\{ t - \frac{1}{\beta}(1 - e^{-\beta t}) \right\}. \quad (8)$$

With CO_2 the primary product, there follows

$$\frac{n_1}{R} = \frac{m k_3 V_1}{\beta V_2 \lambda} \left\{ t + \frac{k_1 m}{k_3 \beta}(1 - e^{-\beta t}) \right\}. \quad (9)$$

For the manometric conditions used, $e^{-\beta t}$ approaches close to zero within a few minutes, so that equations (8) and (9) may then be written

$$n_1 = \gamma(t - 1/\beta) \quad (10)$$

and

$$n_1 = \gamma(t - k_1 m/\beta k_3), \quad (11)$$

where

$$\gamma = \frac{R m k_3}{\beta} \times \frac{V_1}{V_2 \lambda}.$$

If carbonic anhydrase is present in the fluid in sufficient amount to increase greatly the values of k_1 and k_2, then the two equations (10) and (11) reduce to

$$n_1 = \gamma t. \quad (12)$$

If a line be drawn representing moles of CO_2 in the gaseous phase (n_1) for any value of t (assuming a linear relation), this line will have the same slope (γ) in equations (10)–(12). But, if sufficient carbonic anhydrase is added and with H_2CO_3 the primary product of decarboxylation, this line increases to a higher level by $\gamma \times 1/\beta$. The ratio of this increase to the slope is $1/\beta$. It is obvious that such a ratio holds independently of whatever units, besides moles, are used to express the CO_2 content of the gaseous phase, whether μl. or pressures observed directly on the scale of a Warburg manometer. It is also independent of the rate of decarboxylation, provided this is constant or nearly constant throughout the observations and the shaking is adequate.

If CO_2 be the primary product, then on adding carbonic anhydrase the corresponding ratio of the change resulting to the slope is $-k_1 m/\beta k_3$ and the line of n_1 against t falls to a lower level.

These expected theoretical curves are shown in Fig. 1. Here it may be assumed that after some minutes' action the manometer tap is closed and after 3 min. the fluid in the side arm containing carbonic anhydrase (sufficiently strong to increase the k_1 and k_2 values 20 times or more) is mixed with that in the central chamber.

It may be noted that the effect of the carbonic anhydrase is relatively much greater but in an opposite direction when the primary product is free CO_2 than when it is H_2CO_3 (or HCO_3^-).

METHODS

Carboxylase preparation. The method of preparation was that of Green, Herbert & Subrahmanyan (1941).

Dried yeast, very kindly supplied by Prof. Krebs, was mixed with 3 times its weight of 0·067 M-sodium phosphate buffer (pH 7·2) and incubated at 37° for 1 hr. An equal volume of water was added and the mixture centrifuged.

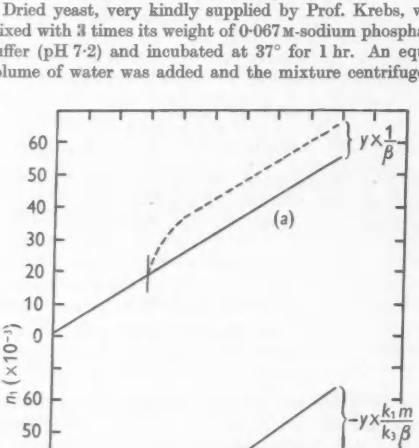

Fig. 1. Diagram showing the full theoretical effect of carbonic anhydrase on the moles of CO_2 liberated into the gaseous phase when carboxylase in a certain strength acts on strong pyruvate solution, and for an initial period produces CO_2 in linear relation with time. pH buffered to 7·0, and temperature 18°. At the beginning of the carboxylase action the manometer is open to the atmosphere, then closed. 3 min. after the closure, carbonic anhydrase is introduced from the side arm, which contains otherwise the same fluid composition as the central fluid. – – –, the presumed course of the CO_2 liberation after adding carbonic anhydrase. ——, the curve when a carbonic anhydrase inhibitor is included in both fluids from the outset. (*a*) This shows the effect of adding carbonic anhydrase, when H_2CO_3 is the primary product of decarboxylation. The ratio of the increase in level ($\gamma \times [1/\beta]$) to the slope (γ) is $1/\beta$. (*b*) This shows the effect of adding carbonic anhydrase when CO_2 is the primary product. The ratio of increase is here $-k_1 m/k_2 \beta$.

The crude extract obtained was mixed with M-calcium acetate and 0·5 M-sodium phosphate buffer (pH 7·2), then centrifuged. The carboxylase was precipitated twice by half-saturation with $(NH_4)_2SO_4$ and then fractionated. The precipitate at 0·4 saturation was discarded and the solution brought to half-saturation. For some experiments this suspension was fractionated a second time, the precipitate at 0·47 saturation being discarded.

A different method of extraction was used for fresh Dublin yeast. The pressed yeast was frozen with solid CO_2 or liquid O_2. After thawing it was centrifuged and the crude extract treated as above. Fractionation with $(NH_4)_2SO_4$ led to a considerable loss of activity, whereas the dried Sheffield yeast gave more satisfactory results, and yielded clearer solutions.

The carboxylase preparation was kept at 0° suspended in half-saturated $(NH_4)_2SO_4$ solution, and used in this form. It retained its activity indefinitely.

When crude yeast extract was used it was prepared freshly before the experiment. The activity may be judged from the following typical result. At 18° and pH 7, 0·25 ml. of the $(NH_4)_2SO_4$ suspension diluted to 2 ml. with 0·25 M-sodium phosphate buffer, on addition of 1 ml. of 2 M-sodium pyruvate yielded 34 μl. CO_2 during the second 2 min.

Carbonic anhydrase preparation. Carbonic anhydrase was prepared from ox blood, following the method of Keilin & Mann (1940). The ethanol-$CHCl_3$ extract is referred to as 'yellow fluid' in the following experiments. This was further purified by dialysing for 3 hr. against running tap water, and precipitated by saturation with $(NH_4)_2SO_4$. The precipitate was collected, dissolved in water and filtered. From 100 ml. 'yellow fluid' 5 ml. of solution containing carbonic anhydrase were thus prepared.

By a microdiffusion method (Conway & McDonnell, 1951; modified procedure of Conway & O'Rourke, 1945) the purified carbonic anhydrase preparation in a dilution of 2000 times (total phosphate buffering of approx. 0·1 M at pH 7·0), and acting on an 0·025 M concentration of $NaHCO_3$, gave an increase of k_1 and k_2 to at least 3·25 times (the maximum observable by the procedure without further dilution).

In the carboxylase experiments it was, however, considered advisable to use the carbonic anhydrase preparation in much more concentrated form owing to possible inhibitory effects; the final dilution was accordingly 34 times. If no additional inhibition occurred this would increase k_1 and k_2 about 200 times.

The protein content of the carbonic anhydrase preparation was determined by heating the acidified solution to 100° for 10 min. The precipitate was collected by centrifuging, washed twice and dried. 2 ml. of solution yielded 0·0162 g. of protein, i.e. 0·81 % (w/v).

Pyruvate solution. 2 M-Sodium pyruvate was prepared from redistilled pyruvic acid (90 %), neutralizing this with 2·4 N-NaOH. The solution was adjusted electrometrically to pH 7·0.

Oxaloacetate solution. 2 M-Sodium oxaloacetate was made immediately before each experiment from crystalline oxaloacetic acid, prepared by the method of Wohl & Osterlin (1901).

α-Ketoglutarate solution. α-Ketoglutarate was prepared by the method of Wislicenus & Waldmüller (1911). The sodium salt (2 M solution) was prepared before each experiment and adjusted to pH 7·0.

Manometric procedure. The following solutions were made up:

(*A*) Carboxylase-buffer preparation at pH 7·0, containing 1 vol. of the carboxylase preparation, 4 vol. of 0·5 M-sodium phosphate buffer (pH 7·0) and 3 vol. of water.

(*B*) Carboxylase-buffer-anhydrase preparation, at pH 7·0, containing 1 vol. of the carboxylase preparation, 4 vol. of 0·5 M-phosphate (pH 7·0) and 3 vol. of the carbonic anhydrase solution.

Such preparations were adjusted, if necessary, to pH 7·0, using the glass electrode.

One flask was set up as follows (after smearing a little white Vaseline around the opening of the side arm as a precaution against any premature mixing of its contents with the main fluid): 2 ml. of solution *A* in central compartment; 0·266 ml. of solution *B* in side arm; 1 ml. of the sodium pyruvate solution (also adjusted to pH 7·0 as described above) was then added to the centre and 0·133 ml. to the side arm simultaneously, noting the time. The flask was attached to the manometer and set shaking in a water bath at 130 oscillations/min. The water bath was at room temperature (normally about 18°) as were all solutions. After 2 min. the tap connecting the manometer to the air was shut and manometric readings taken every 30 sec. After four readings (five or six in some experiments) the side-arm contents were mixed in as quickly as possible (taking no longer than 7 sec. after a little experience) and readings continued for another few minutes. The change in rate of CO_2 production immediately after the addition of side-arm contents indicated the effect of active carbonic anhydrase.

The experiments using inhibited carbonic anhydrase were the same except for the presence of sulphanilamide or thiophen-2-sulphonamide included in the buffer to give concentrations of 0·033 and 0·0014% (w/v), respectively (both in the central and side-arm fluids).

In this type of experiment it was sought to have the composition of the side arm and central fluid as similar as possible at the outset except for the presence of carbonic anhydrase in the side arm.

The dilution of the carboxylase preparation described above was 12 times, for the central and side-arm fluids, and the dilution of the carbonic anhydrase preparation in the final mixed fluids was 34 times.

The only difference in fluid composition between the experiments with active and inhibited carbonic anhydrase was the inclusion in the latter of a small amount of sulphanilamide or thiophen-2-sulphonamide. Such experiments with the inhibited anhydrase gave fully adequate controls, the inhibitor being present in equal concentrations in the central and side-arm fluids.

The activity of the carbonic anhydrase in the mixture in the Warburg flask at the end of such experiments (uninhibited) was tested by a microdiffusion procedure previously mentioned. The buffered mixtures from the flasks were used directly without further dilution.

Manometer cups. As shown in the theoretical section, the relation of the volume of the gaseous phase to that of the liquid phase is of some importance, at least for the theoretical evaluation. In the experiments described the cup had a volume of approximately 8·0 ml. In some of the later experiments a larger cup of the same type was used with a volume of 11·5 ml.

RESULTS

Diffusion effects

In the Theoretical section it was assumed that shaking was sufficient to prevent significant diminution of the gaseous exchange rate. Such limitation produced by diffusion in manometry has been dealt with, for example, by Roughton (1941).

Concerning this effect, if we suppose that carboxylase is acting on strong pyruvate at pH 7·0 under the conditions described in the Methods section and that the slope of n_1 (CO_2 liberated into the gaseous phase) against time is linear but the shaking inadequate, then compared with adequate shaking the effect is similar to an increase in solubility of the gas in the liquid phase or of the λ value as given above. We have carried out a series of experiments to test the adequacy of the shaking on various decarboxylation rates. In these the effect of shaking on various slopes of CO_2 discharge into the gas phase against time was observed. The conditions were the same as for the experiments described above under Methods, except that the contents of the side arm were included in the central chamber from the beginning of the experiment, and different dilutions of the original carboxylase preparations used.

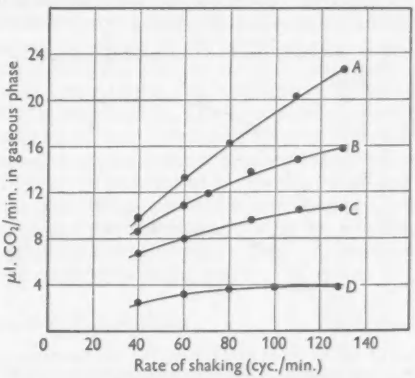

Fig. 2. Effect of shaking rate on CO_2 liberation when carboxylase of different strengths (as in *A*, *B*, *C* and *D*) acts on strong pyruvate at 18°. Conditions described in text.

The results are shown in Fig. 2. It will be seen that with decarboxylation conditions giving a slope of 10 μl./min. with shaking rate of 130 cyc./min., increased shaking will have no appreciable effect. If the evolution of CO_2 were about 13 μl./min. with similar shaking, the curve (between *B* and *C* in Fig. 2) could be extrapolated to give a maximum of about 14 μl./min. Such a slope is of interest here, since in the first group of experiments, described below, showing the formation of H_2CO_3 as the primary product, the average rate of CO_2 discharge into the gaseous phase from 2·5 to 5 min. and without carbonic anhydrase inhibition was likewise 13 μl./min. (The figure from Table 1, summarizing such experiments, is 15 μl./min.; the difference arises from the adjustment of the data by a factor constant for each experiment to give 30 μl. at the 2 min. period.)

With fully adequate shaking the value of $1/\beta$, or the ratio of the increase of CO_2 after carbonic anhydrase to the slope (assuming H_2CO_3 as primary product), is $1/0{\cdot}74 = 1{\cdot}35$ (β being taken from data given under Symbols). However, as 15% of the total mixed fluids has been subjected to the action of carbonic anhydrase in the side arm, the expected $1/\beta$ value is, rather, 1·15. If allowance is made in turn for shaking not being fully adequate, in the form that λ is 1·1 times 0·7 (the figure stated under Symbols) then an observed $1/\beta$ figure of 1·1 may be anticipated. (If the apparent λ value were even as high as $2 \times 0{\cdot}7$, the resulting $1/\beta$ figure would be approximately 0·9.)

With CO_2 the primary product, the expected $k_1 m/\beta k_2$ is 2·55. Allowing for apparent λ values of $1{\cdot}1 \times 0{\cdot}7$, or as high as $2{\cdot}0 \times 0{\cdot}7$, it becomes 2·6 and 2·9 times respectively. Thus, considerable increase in the apparent λ value, due to inadequate shaking, would have but a small effect on $1/\beta$ or on $k_1 m/\beta k_2$.

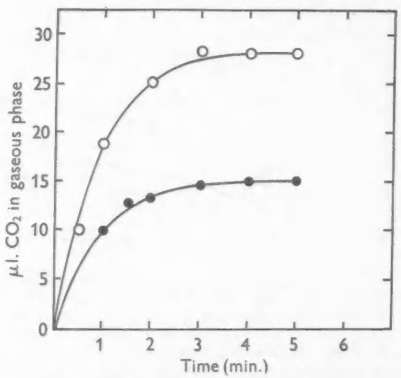

Fig. 3. Curves showing rate at which equilibrium is attained when free CO_2 is liberated from 3·4 ml. of a mixture as described in text. The shaking was 120 cyc./min.

Rate at which a fixed amount of CO_2 *is discharged into the gaseous phase from a mixture buffered at* pH 7·0

0·25 M-Phosphate buffer (pH 7·0) containing a little of the carbonic anhydrase preparation was bubbled with carbon dioxide gas for some time, then diluted with different volumes of the 0·25 M-phosphate solution, containing carbonic anhydrase, to give suitable readings in the next stage. 3·4 ml. of the diluted mixture were quickly introduced into the central chamber of the cup and placed in position. Fig. 3 shows the results of shaking at 120 cyc./min., plotting μl. CO_2 against time. The experiments were suitably controlled using the phosphate solution with carbonic anhydrase, but without CO_2. The results with two different levels of CO_2 (15 and 28 μl.) are given. The rate at which equilibrium is attained is practically independent of the amount discharged, up to the level studied. With a shaking rate of 130 cyc./min., these percentages would be reached a little sooner.

The effect of a pH *difference between side-arm contents and that of central chamber*

With a type of experiment described in the Methods section where the pressure development is observed for some minutes and the contents of the side arm then mixed with the central fluid it was considered advisable to examine first the effect of pH differences deliberately produced, the contents of the chambers being otherwise the same.

Results with a difference of 0·3 pH are here described. The enzyme mixture had the following composition: 4 parts 0·5 M-phosphate buffer at pH 7; 3 parts water; 1 part carboxylase preparation (ammonium sulphate suspension).

Half of this mixture was adjusted electrometrically to pH 6·8 and the other half to 7·1. These

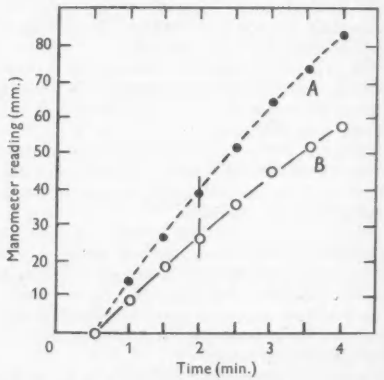

Fig. 4. The curves show the development of pressure during carboxylase action on strong pyruvate, the mixtures buffered to 6·8 or to 7·1 at 18°, when the side-arm contents (0·4 ml.) having the same composition but a somewhat different pH are introduced into the central contents (3·0 ml.). *A*, Central fluid at pH 6·8, side-arm fluid at pH 7·1; *B*, Central fluid at pH 7·1, side-arm fluid at pH 6·8.

were evacuated of gas. 2 ml. of the mixture at pH 6·8 were introduced into the centre and 0·266 ml. of the mixture at pH 7·1 into the side arm. 1 ml. of 2 M-pyruvate at pH 6·8 was added to the centre and 0·133 ml. of 2 M-pyruvate at pH 7·1 simultaneously to the side arm, and the experiment continued as described in the Methods section. After four readings the side-arm fluid was mixed with the centre fluid.

The average of five sets is shown in Fig. 4. The average of five sets using pH 7·1 in the centre and 6·8 in the side arm is also shown.

It will be seen from Fig. 4 that no appreciable change of level or slope occurs on mixing.

This may be explained by the compensatory effect of the pH rise or fall in one fluid as it is mixed with the other.

While, in the other experiments to be described, much care was expended in having the contents of both regions initially at the same pH, it will be seen that even a difference far beyond any that could presumably arise will not explain the results with carbonic anhydrase described below.

Incidentally, such experiments provide additional controls for those which follow, with regard to any effects that might result from tipping in the side-arm contents, as distinct from the action of carbonic anhydrase.

Experimental conditions showing the primary production of H_2CO_3 *(or* HCO_3^-*) in pyruvate decarboxylation*

The experiments were carried out as described under Methods. The carboxylase preparation used was prepared by fractional ammonium sulphate precipitation from the dried yeast supplied by Prof. Krebs. The carbonic anhydrase preparation was used undiluted.

The fluid in the side arm was mixed with that in the centre after four readings at 30 sec. intervals. The first of such readings, 30 sec. after closing the manometer tap, was taken as zero level.

Each of these ten experiments showed the characteristic rise to a new level on mixing with the fluid containing active carbonic anhydrase.

The accompanying experiments with inhibitor were carried out in the same way, except that sulphanilamide was incorporated in the buffer solution of both components to give a concentration of 0·033 % (w/v). In nine out of the ten experiments with inhibitor there was no similar increase of pressure to a new level after tipping in the side-arm contents. The exception would appear to be due to some accident as in a large number of experiments carried out in other series using sulphanilamide (or thiophen-2-sulphonamide) it was not repeated.

It is true that after tipping in the side-arm contents and using such inhibitors the first reading is a little above the level of the original curve but the subsequent readings follow this curve. In Table 1 is given the averages and standard deviations for the individual experiments, and the results of subtracting the means for the experiments with active from those with inactive carbonic anhydrase. To express the degree of variability between the different experiments, and to remove that arising merely from some differences between slopes of n_1 against time (attributable largely to small temperature changes on different days and some difference in the carboxylase action), each reading in a given experiment was multiplied by a factor, this factor bringing the 2 min. reading to 30 μl. (this being near to the mean value in the series).

Table 1. *Time course of decarboxylation experiments*

(This gives the averages for ten sets of decarboxylation experiments, carried out on different days, using the same dried-yeast sample as the source of the carboxylase preparation. The side-arm contents, of the same composition as the fluid in main chamber but containing carbonic anhydrase, were tipped in 2 min. after closing the manometer tap. The reading ½ min. after closing the tap was taken as zero, and, to reduce variability from one experiment to another, the readings for each experiment were multiplied by a factor to bring the 2 min. period to 30 μl. (The actual average rate up to 2 min. of the observation period was approximately 17 μl./min., and towards the end of the observation period about 13–14 μl./min.))

	μl. CO_2		
Min.	Carbonic anhydrase active, tipped in after 2 min.	Carbonic anhydrase inhibited with sulphanilamide, tipped in after 2 min.	Differences between (2) and (3)
(1)	(2)	(3)	(4)
0·5	0±	0±	0±
1	10±0·3	11±0·4	−1±0·5
1·5	21±0·8	22±0·3	−1±0·9
2	30	30	0
2·5	48±0·6	43±1·1	+ 5±1·3
3	60±1·1	53±0·9	+ 7±1·4
3·5	67±1·2	60±1·1	+ 7±1·6
4	76±1·7	68±1·6	+ 8±2·3
4·5	84±1·6	75±1·9	+ 9±2·4
5	93±2·0	83±2·2	+10±2·9
5·5	100±2·3	89±2·3	+11±3·0

The means in cols. 2 and 3 are followed by their S.D.
Col. 4 gives the differences between the means and the S.D. of the differences.

A further series of four sets of experiments was carried out with a similar carboxylase preparation, obtained in turn from another consignment of dried yeast received from Prof. Krebs in March 1949. The results were the same as before. The four sets are summarized in Fig. 5*a*.

The total average value of $1/\beta$ for the sixteen sets of experiments (sixteen with inhibitor and sixteen without) was 0·8.

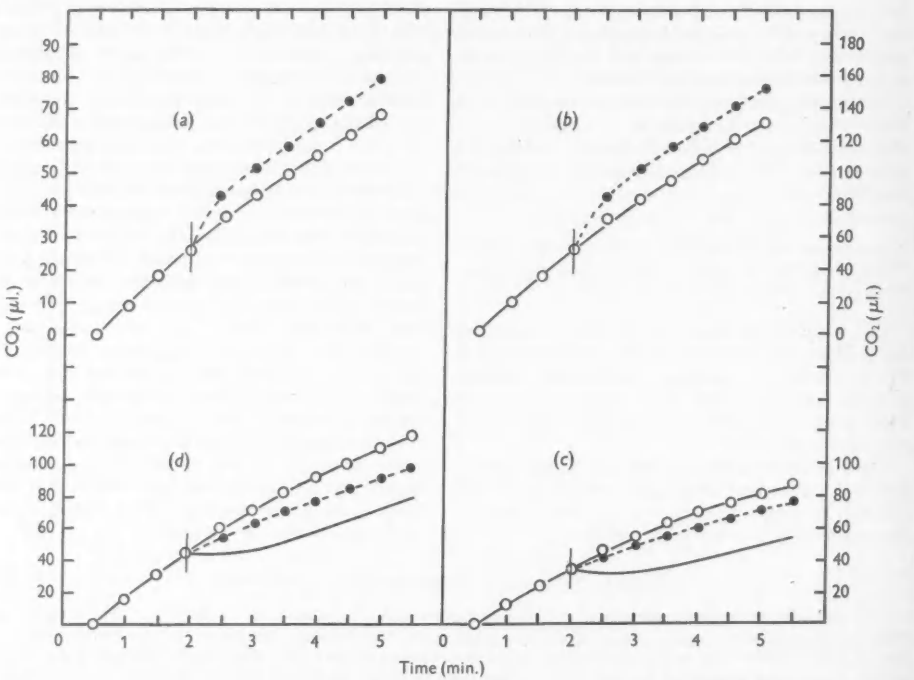

Fig. 5. Effect on CO_2 liberation into gaseous phase when carbonic anhydrase from side arm (0·4 ml.) is introduced into 3 ml. of carboxylase-pyruvate mixture in centre (pH 7·0; 18°). The side-arm fluid has the same composition as the central fluid except for carbonic anhydrase. Conditions described in text; ○—○, curve before and after addition of inhibited carbonic anhydrase; ● - - - ●, the effect when uninhibited carbonic anhydrase is added; ——, theoretical line for CO_2 as full primary product. (*a*), average of four experiments using uninhibited and four companion experiments using the enzyme inhibited with sulphanilamide; (*b*), conditions same as (*a*), but using thiophen-2-sulphonamide as carbonic anhydrase inhibitor (two sets of experiments); (*c*), general conditions same as for previous diagrams, but 'yellow fluid' was used as the carbonic anhydrase preparation and a direct water extract of dried yeast as the carboxylase preparation; (*d*), conditions as for (*a*), but with a more impure carboxylase preparation from a different yeast: for details see text.

Two sets were in turn carried out using thiophen-2-sulphonamide as the inhibitor, as described under Methods, and are shown in Fig. 5*b*. (For the data in Fig. 5 no factors were used to adjust to a common level at the 2 min. period.)

It will be seen from a comparison of Fig. 5*a* and *b*, with Fig. 1*a* that the results agree closely with the theoretical form, when the primary product is not free carbon dioxide. While the trend of pressure production is not exactly linear, it is nearly linear and after the increase to a higher level the further development of pressure follows practically parallel with the original curve.

As considered above, the expected value with H_2CO_3 as the primary product is about 1·0. The difference may not be significant, as the theoretical calculation of β depends on data which are not known with exactitude. It shows that at least much the greater fraction of the primary product was H_2CO_3.

Each of the sixteen experiments showed a similar rise of pressure to a new level when the content of the side arm containing active carbonic anhydrase was mixed into the main chamber.

It was apparent to us that with the conditions described such results could be indefinitely repeated with carboxylase preparations obtained from the yeast samples received in November and March and prepared as described according to the procedure of Green *et al.* (1941).

Experimental conditions in which the primary product of decarboxylation was chiefly CO_2

In May 1949 we sought, as described in the Methods section, to prepare from a Dublin pressed yeast a carboxylase of purity similar to that from the dried Sheffield yeast. The preparation in this case was not very successful and gave a turbid instead of a clear solution, although on previous occasions we had obtained purer preparations from a similar source. Using such a preparation and the carbonic anhydrase preparation described under Methods, we found that after tipping in the side-tube contents the pressure production was at a somewhat lower level than with the inhibited mixture. The average results of two sets of experiments are given in Fig. 5*c*, in which the theoretical line for CO_2 as the full primary product is also given.

These experiments were also carried out using a water extract of the dried Sheffield yeast as the carboxylase solution, and the 'yellow fluid' as the carbonic anhydrase preparation.

Such solutions were used by Krebs & Roughton (1948) and the result at 18° was similar to theirs at 15°.

We used the solutions prepared as they described but with our technique of adding the side-arm contents during the pressure development. The mixture in side arm and central region was made in the same proportions as described under Methods, but the water extract of the dried Sheffield yeast was used as the carboxylase and the 'yellow fluid' as the carbonic anhydrase preparation.

The results of two sets of experiments are given in Fig. 5*d*. After mixing with the active carbonic anhydrase the curve of pressure falls to a lower level. The conclusion may be drawn that the primary product under such conditions is mainly free CO_2.

The results differ in some important respects from the theoretical expectation if the primary product is altogether CO_2. Thus, the curve of pressure after the active carbonic anhydrase does not tend to run parallel with that using the inhibited enzyme. Also, the ratio of the difference between the two curves to the slope, even taking the smaller slope, with active carbonic anhydrase and after 4 min., is only 1·5, the expected value with CO_2 as the primary product being about twice this figure. The divergence of the curve from that theoretically expected if CO_2 were the full primary product is shown in Fig. 5*d*.

From this it may be concluded that CO_2 was produced directly only in some preponderance over H_2CO_3 or its ion.

Experiments with oxaloacetate and α-ketoglutarate

A series of experiments was conducted with sodium oxaloacetate as the substrate in the same concentration as pyruvate and with carboxylase and carbonic anhydrase prepared as described under Methods. The first series (four experiments) is summarized in Fig. 6*a*. It will be seen that on mixing in the side-arm contents the result was an unexpected change of slope even in the presence of sulphanilamide.

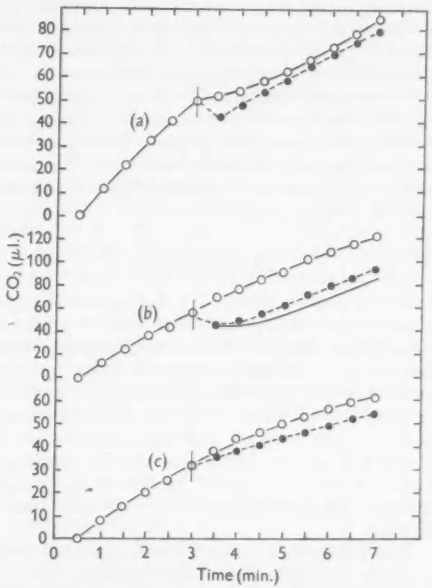

Fig. 6. Results of experiments with oxaloacetate. O—O, curve before and after addition of inhibited carbonic anhydrase; ●—●, effect when uninhibited carbonic anhydrase is added; ——, theoretical line for CO_2 as full primary product. (*a*) Similar conditions to Fig. 5*a*, except oxaloacetate used as the carboxylase substrate. Average of four sets inhibited and four sets uninhibited. (*b*) Similar to (*a*), but diluted blood used as the carbonic anhydrase preparation. (0·1 ml. of twice-diluted rabbit whole blood in side-arm contents; final blood dilution 1:68 in the mixed fluids.) Average of two sets uninhibited and two sets inhibited. (*c*) Similar to (*b*), except that α-ketoglutarate was used as the carboxylase substrate. Average of two sets inhibited and two sets uninhibited.

We carried out a further series using laked blood as the source of the carbonic anhydrase. The blood had a final dilution of 68 times in the mixed fluids. The results are summarized in Fig. 6*b* (from two experiments) and show a typical picture of the course of pressure development with the primary product chiefly CO_2 and not H_2CO_3 or its ion. The

comparison may here be drawn between Fig. 6*b* and the theoretical Fig. 1*b*. Thus, the difference in tension produced on mixing with the active carbonic anhydrase is rapidly developed and the further rise of pressure is practically parallel with the pressure developed in the presence of the inhibited carbonic anhydrase. Further, this latter continues the curve of pressure up to the mixing point without any deviation on mixing. The $k_1 m/k_2 \beta$ value approached 2·1 and the expected value for full CO_2 production as primary product is about 2·5 (not allowing for inadequacy of shaking). In all such experiments this is the nearest approach we have obtained to a decarboxylation with CO_2 only as the primary product, and with typical theoretical form.

With sodium α-ketoglutarate the results, using diluted blood as the source of the carbonic anhydrase, also showed that CO_2 was the main primary product (Fig. 6*c*), but the depression of the pressure level is far less than the expected figure if CO_2 were the only primary product.

DISCUSSION

From the results described it will appear that preparations of carboxylase from a dried yeast obtained from Sheffield (following the procedure of Green *et al.* 1941) decarboxylated strong pyruvate solution at pH 7·0 with a primary production of carbonic acid (or HCO_3^-). The increase in pressure on mixing in the carbonic anhydrase approached the theoretical amount, but the results are compatible with the view that a small fraction of the primary product of decarboxylation was free carbon dioxide.

In any assessment of such experiments and results as those described above, the following two facts should be noted. First, when carboxylase is acting on strong pyruvate (buffered at pH 7·0 with phosphate) in the main compartment of a Warburg manometer, the curve of pressure is not appreciably changed on tipping in side-arm contents of the same composition to the main fluid and under the experimental conditions described. Secondly, the curve of pressure is not appreciably changed when the side-arm contents, otherwise of the same composition as that in the main chamber, contain carbonic anhydrase inhibited with sulphanilamide or thiophen-2-sulphonamide (the inhibitor being present in both compartments in the same concentration).

Such controls appear to be entirely adequate and those with sulphanilamide or, alternatively, thiophen-2-sulphonamide (two of the inhibitors studied by Krebs (1948)) were carried out with each experiment in which the effect of carbonic anhydrase on the carboxylase system was examined.

On the other hand, preparations of carboxylase can be obtained which decarboxylate strong pyruvate with free carbon dioxide as the major product, agreeing, at least qualitatively, with the observations of Krebs & Roughton (1948). Thus, we used their carboxylase preparation of a water extract of dried Sheffield yeast and the 'yellow fluid' as the carbonic anhydrase preparation, but with the procedure described in this paper.

The addition of the active carbonic anhydrase caused a fall in rate of carbon dioxide production, compared with the control curve with inhibited carbonic anhydrase. The effect, however, was much less than the theoretical requirement if carbon dioxide were the full primary product in decarboxylation (Fig. 5*d*). In this connexion the theoretical curve may be compared with Fig. 6*b*, showing the results with oxaloacetate, and diluted blood as the source of the carbonic anhydrase.

Such results as a whole may be best interpreted by the view that decarboxylation takes place through two paths, the primary product being a mixture of free carbon dioxide and of carbonic acid, the preponderance of one or the other depending, apparently, on the total conditions. This conclusion would not, of course, be invalidated if some special condition (including even the use of the purest enzymes) was found which showed a considerable preponderance of the primary pathway through carbon dioxide.

In the experiments with 'yellow fluid' as the direct source of carbonic anhydrase and the water extract of dried yeast, the question may be raised whether the change in the decarboxylation of pyruvate was due to relative impurities in the carboxylase extract or the 'yellow fluid'. Since a similar result (Fig. 5*c*) was obtained with a relatively impure carboxylase preparation from a Dublin yeast and the carbonic anhydrase preparation described under Methods, it would appear that the carboxylase preparation could furnish the operative impurity. At the same time we leave it at present an open question as to whether a similar result might not arise from the use of a relatively crude source of carbonic anhydrase.

Our interest in such decarboxylation lay chiefly in its being a possible direct source of H^+ ions, as, for example, in the remarkable H^+ and K^+ exchange in fermenting yeast (Conway & O'Malley, 1943, 1946). Here, under suitable conditions, HCO_3^- accumulated in the yeast cells in nearly quantitative relation to the H^+ ions exchanged (Conway & Brady, 1947). Such facts, however, have been better interpreted by an oxidation-reduction theory of the acidic production (Conway & Brady, 1948; Conway, 1949; Conway, Brady & Carton, 1950). The change of interpretation is not related to the question of the path of decarboxylation.

SUMMARY

1. The derivation is given of equations (previously stated by Conway & O'Malley, 1948) from which the full theoretical effect of carbonic anhydrase on the increase of manometric pressure during decarboxylation may be calculated. It is assumed that the decarboxylation, as of strong pyruvate, is a linear function of the time over the observation period. Such equations may be further developed for special conditions where the decarboxylation is a definite, but non-linear function of the time.

2. A carbonic anhydrase preparation (Keilin & Mann, 1940) was introduced from the manometer side arm, being present there in fluid having otherwise the same composition as the central fluid. This contained carboxylase prepared according to the procedure of Green *et al.* (1941) acting on 0·67 M-pyruvate at pH 7·0 and 18°. The production of pressure was nearly linear over the time of observation. With the introduction of active carbonic anhydrase there was a rapid rise of pressure which after a few minutes proceeded practically parallel with the previous curve, or with that using inhibited carbonic anhydrase.

3. The mean increase of pressure level, after carbonic anhydrase action, approaches but does not quite reach the expected figure when carbonic acid (or HCO_3^-) is the primary product of decarboxylation.

4. Using a crude water extract of dried yeast as the carboxylase preparation and the 'yellow fluid' as that of carbonic anhydrase, there was a fall of pressure on introducing the latter. This is in qualitative agreement with the experiments of Krebs & Roughton (1948) and shows a primary production of carbon dioxide. The degree of fall was only a fraction of that to be expected if carbon dioxide was the sole primary product of the carbon dioxide system.

5. Experiments were also carried out with strong concentrations of α-ketoglutarate and oxaloacetate, used as carboxylase substrates. With the introduction of the carbonic anhydrase preparation there occurred a small fall of pressure indicating carbon dioxide as mainly the primary product, but the curves of pressure were not regular. Using diluted blood as the source of the carbonic anhydrase, the result with oxaloacetate was similar to the theoretical picture when carbon dioxide is the primary product of decarboxylation. Similar results with α-ketoglutarate indicated a primary production mainly of carbon dioxide, but to a lesser degree than with oxaloacetate.

6. The conclusion may be drawn that in the decarboxylation of keto-acids by yeast carboxylase, carbon dioxide and carbonic acid (or HCO_3^-) are both primary products, but one or the other may predominate depending on the total conditions.

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Studies on β-Glucuronidase

4. THE PURIFICATION AND PROPERTIES OF OX-LIVER β-GLUCURONIDASE

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While a fairly extensive characterization of the preparation and properties of ox-spleen β-glucuronidase has been recorded (Fishman, 1939*a*, *b*; Graham, 1946; Mills, 1948; Mills, Paul & Smith, 1949; Bernfeld & Fishman, 1950*a*; Mills, Paul & Smith, 1953), the available data for liver glucuronidase is confined to the work of Sarkar & Sumner (1950). These authors, using low-temperature dioxan fractionation for the purification of ox-liver β-glucuronidase, achieved a 6000-fold purification of the enzyme. Their method of expressing purity, however, is based on an initial expression of activity per gram of fresh tissue, this being compared with a final activity in terms of mg. dry weight of enzyme. It is more accurate to enumerate both values in terms of a common factor, namely protein nitrogen, in which case a 900-fold purification was achieved. By a combination of metallo-protein interactions, organic solvent precipitation and ammonium sulphate fractionation it has been found possible, in the present work, to achieve a purification of ox-liver β-glucuronidase equivalent to that obtained by Sarkar & Sumner (1950). A study of the properties of the purified material does not support the observation of Sarkar & Sumner (1950) that the β-glucuronidase of ox liver is a single enzyme with a single pH optimum at pH 4·5 in acetate buffer, and data are presented to show that the liver enzyme is complex and analogous in many respects to that reported by Mills (1948) and Mills *et al.* (1953) for ox-spleen β-glucuronidase. Preliminary reports of this work have already appeared (Smith, 1951; Mills & Smith, 1952).

METHODS

Assay of β-glucuronidase preparations. Phenolphthalein β-D-glucuronide, prepared biosynthetically by the method of Talalay, Fishman & Huggins (1946), was used as substrate, and the method of assay was that described by Mills *et al.* (1953). The unit of enzyme activity is that which liberates 1 μg. phenolphthalein in 1 hr. from 1 mM-phenolphthalein glucuronide at pH 4·5 and 38°.

When studying inhibitory substances, the enzyme digests were made up to contain 0·5 ml. buffer solution, 0·2 ml. enzyme solution, 0·1 ml. water or inhibitor solution and 0·2 ml. substrate solution.

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Unless otherwise stated, the following buffers were employed: for the pH range 3·4–6·0, 0·2 M-acetate buffers, and for the pH range 6·0–7·0, the veronal-acetate buffers of Michaelis (1931).

Filter-paper electrophoresis. This was carried out by the method of Mills & Smith (1951).

pH *measurements.* These were made with a glass electrode and either a Muirhead, Tinsley or Marconi pH meter.

Protein N. This was estimated by the method of Ma & Zuazaga (1942), using the Markham (1942) distillation unit.

EXPERIMENTAL AND RESULTS

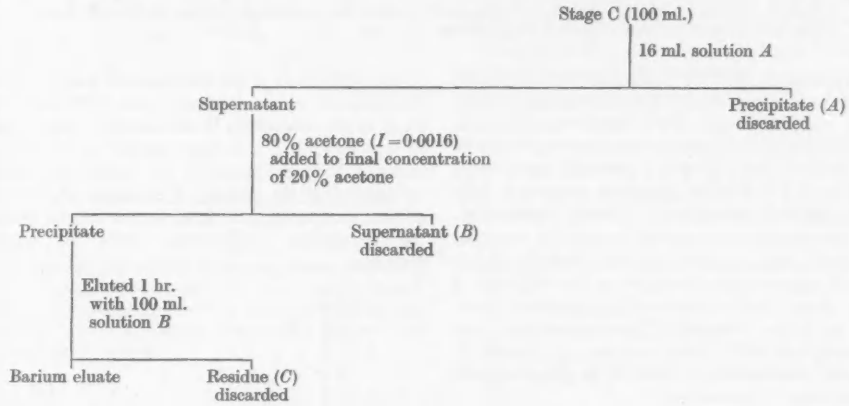
A recent publication of Cohn *et al.* (1950) on the use of the interactions of different proteins with bivalent cations to separate the protein components of human plasma, suggested the possibility of using such metallo-protein complexes in the purification of β-glucuronidase. The following purification scheme has been devised, taking as starting material the stage C fraction described by Mills (1948) in studies on ox-spleen β-glucuronidase.

Finely minced ox liver was extracted twice with 2 vol. acetone and the filter cake dried in air at room temperature. The powder so obtained was extracted with water, the extract adjusted to pH 5·0 with acetic acid, buffered with acetate buffer and autolysed at 38° for 4 hr. The voluminous precipitate so formed was removed in the Sharples centrifuge and solid $(NH_4)_2SO_4$ stirred into the solution to 60% saturation, followed by equilibration at 0° for 18 hr. The precipitate was then removed by filtration, using Standard Super-Cel (Celite 519A, Johns-Manville Corporation), the filter cake extracted with water and the extract dialysed, this extract constituting the stage C of Mills's (1948) original purification.

Further purification can be conveniently divided into three stages.

Stage 1. Initial treatment of the Stage C solution with zinc acetate solution results in the precipitation of a large percentage of inactive protein. If this is removed by centrifugation, glucuronidase can be precipitated completely from the supernatant by the addition of acetone to a final concentration of 20%. This is in contrast to the higher value of 45% acetone required for precipitation of the enzyme in the absence of Zn^{2+}. Elution of the precipitated material with a 15% acetone solution containing Ba^{2+} results in a selective extraction of β-glucuronidase yielding the so-called 'barium eluate'. The procedure is represented in Fig. 1. The percentage distribution of the enzyme obtained by the above fractionation is shown in Table 1.

The success of stage 1 is dependent on a number of factors, control of which is important for reproducibility of results. These are: (1) The initial ratio of enzyme activity to protein concentration in the stage C solution is important, and the solution should be adjusted to a value of 2000–4000 units of activity/mg. protein N to avoid undue loss of enzyme in the initial zinc precipitation. (2) Strict temperature control is essential throughout as small variations in temperature may lead to critical disturbances in the system. A temperature of 0° was employed throughout stage 1 of purification. (3) The presence of Ba^{2+} allows the precipitated enzyme to be eluted within the acetone range of 15–20% to give the barium eluate. A complete recovery of glucuronidase was not effected, some 17% remaining in the residue. This was dependent on the time period of elution. Extraction for 1 hr. allows the maximum separation of enzyme relative to the amount of inactive protein simultaneously eluted. With longer extraction periods almost all of the enzyme can be recovered but with a decrease in purity.


Stage C (100 ml.)
16 ml. solution A
Supernatant
Precipitate (A) discarded
80% acetone (I = 0·0016) added to final concentration of 20% acetone
Precipitate
Supernatant (B) discarded
Eluted 1 hr. with 100 ml. solution B
Barium eluate
Residue (C) discarded

Solution *A*: 5·48 g. zinc acetate dihydrate per 100 ml. water.
Solution *B*: per 100 ml. water: 0·26 g. barium acetate, 2 ml. M-sodium acetate, 0·73 ml. M-acetic acid, 15 ml. acetone. pH 5·5–5·6.

Fig. 1. Scheme of stage 1 of purification of ox-liver β-glucuronidase.

Table 1. *Percentage distribution of ox-liver β-glucuronidase during purification*

	Distribution of enzyme (%)
Stage 1	
Barium eluate	60
Precipitate (*A*)	15
Supernatant (*B*)	0
Residue (*C*)	17
Stage 2 (from barium eluate)	
1·8M-$(NH_4)_2SO_4$ fraction	43
1·6M-$(NH_4)_2SO_4$ fraction	30
1·4M-$(NH_4)_2SO_4$ fraction	15
1·2M-$(NH_4)_2SO_4$ fraction	2

Stage 2. Following dialysis of the barium eluate at 0°, glucuronidase was precipitated from solution by adding solid $(NH_4)_2SO_4$ to 60% saturation. Celite 519 A (2%, w/v), was added to adsorb the enzyme which, after filtration, was eluted from the Celite with four 25 ml. portions of 1·8M-$(NH_4)_2SO_4$ solution. The filter cake was further extracted with similar portions of 1·6, 1·4 and 1·2M-$(NH_4)_2SO_4$ solutions. The combined eluates of each individual fraction were adjusted to a final concentration of 3M with solid $(NH_4)_2SO_4$, the precipitate spun off at 0° and dissolved in the minimum volume of water (generally 3 ml.).

90% of the total glucuronidase present in the barium eluate can be recovered by the above procedure with the percentage distribution shown in Table 1.

Most of the enzyme activity was associated with the 1·8M eluate, but this was discarded in view of its high content of inactive protein. The 1·6 and 1·4M fractions were highly active (80000–100000 units of activity per ml.), and were associated with low protein contents.

Stage 3. A large percentage of β-glucuronidase was precipitated from solution on dialysis of the combined 1·6 and 1·4M-$(NH_4)_2SO_4$ fractions obtained in stage 2. An investigation of the effects of controlled dialysis on the purification of the enzyme was considered and two techniques were employed.

(*a*) A fairly exhaustive dialysis was required to precipitate the enzyme from solution, and inactive material could be removed initially by a short dialysis period (24 hr.). A more prolonged dialysis of the supernatant (3–4 days) against several changes of distilled water precipitated the enzyme which, after centrifugation and elution with 3 ml. 1% NaCl solution, yielded a glucuronidase preparation containing 200000 units of activity per mg. protein N.

(*b*) Alternatively, the glucuronidase was first precipitated from solution by a 3- to 4-day dialysis against distilled water, followed by extraction of the centrifuged precipitate with 6–8 ml. of a 1% NaCl solution. Dialysis of the extract reprecipitated the enzyme, which on a further elution with 3 ml. of 1% NaCl solution gave an enzyme preparation equivalent in purity to that obtained by method (*a*).

Table 2. *Purification of ox-liver β-glucuronidase*

	Units*/ml.	Volume (ml.)	Total units*	Purity†	Yield (%)
Liver mince	6 000	—	—	240	—
Stage *C*	20 000	100	2×10^6	2 500	100
Barium eluate	12 000	100	$1{\cdot}2 \times 10^6$	14 000	60
1·6 M-$(NH_4)_2SO_4$ fraction	80 000	4	32×10^4	—	16
1·4 M-$(NH_4)_2SO_4$ fraction	60 000	3	18×10^4	—	9
Stage 3 purified material	20 000	5	10×10^4	200 000	5

* Unit of activity = 1 μg. phenolphthalein liberated/hr. under the conditions specified in Methods section.
† Purity expressed in terms of activity/mg. protein N.

A temperature of 0° or less must be maintained throughout dialysis. When dialysis was carried out against running water (12°), rapid denaturation occurred with an overall loss in enzyme activity of 35 % over a period of 8 hr., whereas losses were negligible at 0°. A short period of extraction with 1 % sodium chloride solution (15 min.) preferentially elutes the enzyme from the precipitate obtained on dialysis; with longer extraction periods, higher yields of enzyme are obtained at the expense of purity. Quantitative details of the purification are shown in Table 2. Starting from minced ox liver containing 240 units of activity per mg. protein N, an overall purification of 800-fold is obtained with 5 % recovery of the enzyme.

Properties of the purified material

The purified β-glucuronidase is a pale, straw-coloured solution. Solutions containing 200 000 units of activity per mg. protein N appeared to be relatively unstable, and when stored at 0° for 2 days a 25 % loss in activity was apparent. After this there was no further loss in activity for at least 2–3 weeks at 0°, and the properties of the enzyme were accordingly obtained from this more stable material (150 000 units per mg. protein N).

Isoelectric point of ox-liver β-glucuronidase. The isoelectric point of purified ox-liver β-glucuronidase was determined by estimating the mobility of the enzyme on filter-paper strips at various pH values from 4·0 to 8·0. Crystalline bovine serum albumin (Armour) was examined at the same time to determine the accuracy of the method. The results of this experiment are shown in Fig. 2, which shows that the isoelectric point of β-glucuronidase is at pH 4·85 and that for crystalline bovine serum albumin is at pH 4·65. This latter value corresponds well with the accepted value of pH 4·7.

Amino-acid constitution of purified ox-liver β-glucuronidase. To 2 ml. enzyme solution containing 0·22 mg. protein N (purity = 150 000 enzyme units/mg. protein N) concentrated hydrochloric acid was added to a final concentration of 6 N. The enzyme was hydrolysed by heating for 18 hr. at 110° in a sealed tube. Hydrochloric acid was evaporated off *in vacuo* over solid potassium hydroxide and the residue dissolved in 0·2 ml. distilled water. A two-dimensional chromatogram was obtained with 50 μl. of the extract on Whatman no. 1 paper, using as solvents: (*a*) *n*-butanol-acetic acid (40 % *n*-butanol, 10 % glacial acetic acid and 50 % water by volume) and (*b*) phenol. The result showed the presence of seventeen amino-acids in the purified β-glucuronidase preparation with no unusual features.

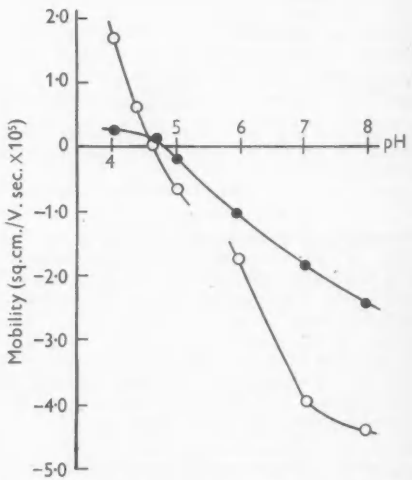


Fig. 2. Electrophoretic migration of purified ox-liver β-glucuronidase (●—●) and crystalline bovine serum albumin (Armour) (○—○) on filter-paper strips. Acetate buffers, pH 4–5, and phosphate buffers, pH 6–8. $I = 0{\cdot}05$.

pH-*Activity curve.* A typical pH-activity curve for the purified enzyme is shown in Fig. 3. While the enzyme appears to be stable over a wide pH range, the presence of a number of well-defined optima in a preparation of such high purity is suggestive of the complexity of the system. The pH-activity curve in the region of pH 5·5–7·0 was re-investigated using phosphate-citrate buffer and the presence of an optimum at pH 6·3 confirmed. Although the

activity curve presented may be considered representative of the purified enzyme, different preparations showed individual variation in the proportionality of activity at the pH maxima. It is interesting to note that all highly purified enzyme

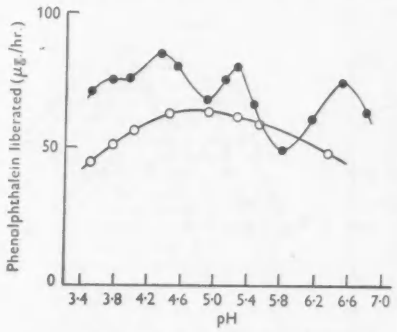


Fig. 3. (●—●), pH-activity curve of purified ox-liver β-glucuronidase. Purity = 150000 units per mg. protein N. (○—○), pH-activity curve of above preparation after standing at 0° for 3 weeks. These curves are not to be related quantitatively.

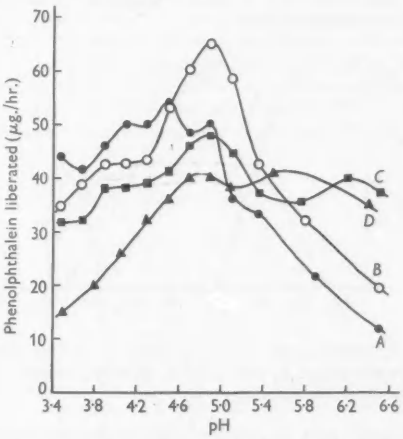


Fig. 4. pH-Activity curves of purified ox-liver β-glucuronidase at various concentrations of phenolphthalein glucuronide. *A*, 0·25 mM; *B*, 0·5 mM; *C*, 1·0 mM; *D*, 4·0 mM.

preparations when allowed to stand for any length of time (3 weeks at 0°) showed a gradual change in the pH-activity curve from that of well defined optima to the plateau-shaped curve shown in Fig. 3. The quantitative relationship between these two curves has not been investigated.

Fig. 4 shows the variation in shape of the pH-activity curve of a preparation of purified ox-liver β-glucuronidase assayed at different substrate concentrations. These changes in pH-activity curve with varying substrate concentration, which are analogous to those already reported for ox-spleen β-glucuronidase (Mills *et al.* 1953), indicate that in all probability we are again dealing with a mixture of β-glucuronidases with different pH optima and differing substrate affinity.

The effect of deoxyribonucleic acid (DNA) and other compounds on the activity of ox-liver β-glucuronidase

Bernfeld & Fishman (1950*b*) claim to have shown that calf-spleen β-glucuronidase preparations contain a thermostable coenzyme which dissociates from the parent molecule on dilution. While the nature of this coenzyme is still unidentified, it has been shown by the above authors that the action of this coenzyme may be duplicated by adding deoxyribonucleic acid (DNA) to the system.

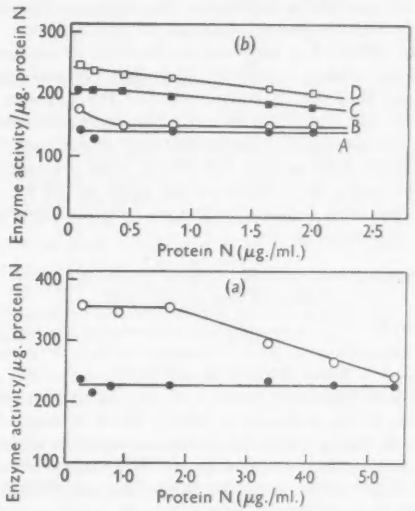


Fig. 5. (*a*) Effect of dilution on the enzyme activity to protein ratio (*E*/*P*) in the absence (●—●) and the presence (○—○) of 0·06% DNA. Enzyme assayed at pH 4·5. (*b*) Effect of dilution on the enzyme activity to protein ratio (*E*/*P*): *A*, in the absence and *B*, in the presence of 0·2% starch; *C*, 0·1% gum acacia and *D*, 0·05% crystalline bovine serum albumin (Armour). Enzyme assayed at pH 4·5.

Studies on purified ox-liver β-glucuronidase in the present work, however, do not indicate that DNA functions as a coenzyme-like substance. Enzyme activity was determined at varying dilutions in the presence and absence of DNA (final concentration, 0·06%; prepared from calf thymus by the method of Mirsky & Pollister, 1946). If glucuronidase

activity is dependent on a coenzyme dissociating on dilution, the ratio of enzyme activity to protein concentration (E/P) will drop greatly with dilution as observed by Bernfeld & Fishman (1950*b*) for calf-spleen β-glucuronidase, and a plot of the E/P ratio against protein concentration should take the form of an S-shaped curve in accordance with the dissociation expression:

$$\frac{(P-E)\,(C-E)}{E}=K,$$

where P = total protein, C = total coenzyme, E = active enzyme, P/C constant and K a constant. In the presence of DNA, the activity of β-glucuronidase should be proportional to the protein concentration. Such a plot is presented in Fig. 5*a* for purified liver β-glucuronidase assayed at pH 4·5. Fig. 5*a* shows that a plot of enzyme activity to protein concentration is linear at all dilutions down to a protein concentration of 0·05 μg. protein N per ml. Such results provide no indication of a coenzyme dissociating from the parent molecule at high dilutions.

Added DNA is an effective activator of ox-liver β-glucuronidase at pH 4·5 (Fig. 5*a*). It was considered that this activating effect of DNA need not be specific and a number of other high molecular weight compounds were investigated in this respect. Albumin, gum acacia and starch were arbitrarily chosen, and their effects on the assay at pH 4·5 of purified liver β-glucuronidase at varying dilutions are shown in Fig. 5*b*.

Inhibition of ox-liver β-glucuronidase by DNA, suramin and alginic acid

While DNA is an effective activator of ox-liver β-glucuronidase at pH 4·5 (acetate buffer), a phenomenon of equal interest is the inhibitory action of DNA at lower pH values. A typical pH-activity curve in the presence of 0·06 % DNA is shown in Fig. 6. Sharp inhibition was apparent below pH 4·2 and activation above this pH. The shape of the curve altered to give a single optimum at pH 4·5–5 with a rise in activity in this region.

An interesting comparison may be made between the effect of added DNA and the action of suramin on certain enzymes. Wills & Wormall (1950) and Wills (1952) have shown that suramin, a polysulphonic acid, inhibits many enzymes on the acid side of their isoelectric points. When percentage inhibition was plotted against pH in such instances, a sharp drop was evident in the inhibition-pH curve, 50 % inhibition occurring at or about the isoelectric point of the enzyme. A similar sharp drop is evident in the inhibition-pH curve of liver glucuronidase in the presence of DNA, and Fig. 7 compares the effect of both DNA and suramin on liver glucuronidase activity in terms of an inhibition-pH graph. Although DNA and suramin are dissimilar in the pH range over which inhibition occurs, their effects are none the less comparable in many respects, and suramin has also been shown to exert a similar activating effect to that of DNA in the higher pH range (Fig. 6).

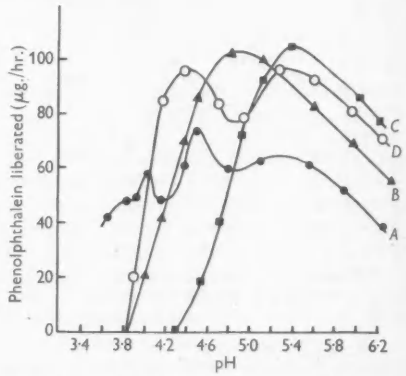


Fig. 6. Effect of 0·06 % DNA, 10^{-3} M-suramin and 0·1 M-phthalate buffers on the pH-activity curve of purified ox-liver β-glucuronidase: *A*, control in 0·1 M-acetate buffer; *B*, plus 0·06 % DNA; *C*, plus 10^{-3} M-suramin; *D*, assays in 0·1 M-phthalate buffers.

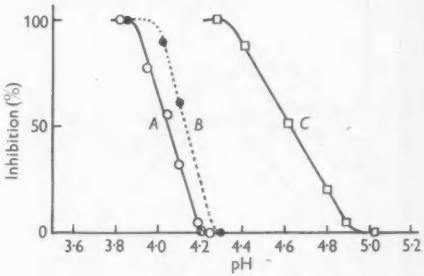


Fig. 7. Effect of pH on the inhibition of ox-liver β-glucuronidase by: *A*, 0·06 % DNA; *B*, 0·06 % alginic acid and *C*, 10^{-3} M-suramin.

Alginic acid is another high molecular weight compound whose inhibition of glucuronidase depends on pH, and the inhibition-pH curve for this compound is also shown in Fig. 7.

Effects of organic acids on ox-liver β-glucuronidase activity

Morrow, Carroll & Greenspan (1951), in a study of the glucuronidase activity of inbred mice, referred to the single pH optimum obtained in the assay of mouse-kidney homogenates. A most surprising feature of the activity curve was the total absence of activity below pH 3·8, the curve rising sharply from

Table 3. *The effect of structurally related organic acids on ox-liver β-glucuronidase activity*

(All compounds assayed at a final concentration of 10^{-2}M. Activity determined at pH 3·4, 4·5 and 5·2 (0·2M-acetate buffers). Enzyme purity = 170000 units activity/mg. protein N.)

Compound	K_a*	Activation (+) or inhibition (−) (%) pH 3·4	pH 4·5	pH 5·2
Benzoic acid	$6·5 \times 10^{-5}$	+37	+6	+17
2-Hydroxybenzoic acid	—	−100	−4	+9
3:5-Dihydroxybenzoic acid	$9·1 \times 10^{-5}$	−9	0	0
2:6-Dihydroxybenzoic acid	$5·0 \times 10^{-2}$	−100	+5	+26
3:4-Dihydroxybenzoic acid	$3·3 \times 10^{-5}$	0	0	0
2:5-Dihydroxybenzoic acid	$1·3 \times 10^{-3}$	−100	−85	−76
3:4:5-Trihydroxybenzoic acid	—	−29	−40	−19
Phthalic acid	1st. H $1·3 \times 10^{-3}$	−100	+50	+47
*iso*Phthalic acid	—	−23	+50	+45
4:6-Dihydroxy*iso*phthalic acid	—	−89	+48	+40
2:5-Dihydroxyterephthalic acid	—	−100	+57	+41
Benzenesulphonic acid	—	+56	+25	+28
Benzoic acid 3:5-disulphonic acid	—	−100	+55	+93
Salicylsulphonic acid	—	−100	−4	+8
Resorcinol	—	0	0	0
Alanine	—	0	0	0
Phenylalanine	—	+62	+14	−70
Tyrosine	—	+47	+10	−100
2:5-Dihydroxyphenylalanine	—	−100	−13	+19

* From *Dictionary of Organic Compounds* (1943). Ed. by Heilbron, I. H. and Bunbury, H. M. London: Eyre and Spottiswoode.

zero activity at this pH to the optimum around pH 4·5. It was noted that phthalate-hydrochloric acid and phthalate-sodium hydroxide buffers were used by Morrow *et al.* (1951), and it was considered that the lack of activity in the lower pH range was probably due to specific inhibition of enzyme

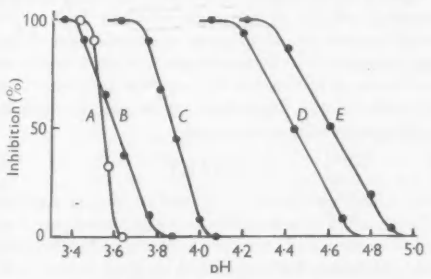


Fig. 8. Effect of pH on the inhibition of ox-liver β-glucuronidase by: *A*, 10^{-2}M-benzoic acid 3:5-disulphonic acid; *B*, 10^{-2}M-2:5-dihydroxyterephthalic acid; *C*, 10^{-2}M-phthalic acid; *D*, 2×10^{-3}M-2-naphthol-3:6-disulphonic acid; *E*, 10^{-3}M-suramin.

activity by phthalic acid. This was confirmed by assaying the purified enzyme in both phthalate and acetate buffers. Reference to Fig. 6 shows the marked inhibitory effect of phthalate in the lower pH range and the considerable activation above pH 4·0. Phthalate increased the activity at the optima at pH 4·5 and 5·2.

The inhibition of ox-liver β-glucuronidase activity by a number of structurally related organic compounds was studied. Inhibition was determined at pH 3·4, 4·5 and 5·2 in acetate buffer. All acids were neutralized prior to assay with N-NaOH. The results are summarized in Table 3.

In Fig. 8 is shown a comparison of the pH-inhibition curves of glucuronidase for a number of the compounds listed in Table 3.

DISCUSSION

While Sarkar & Sumner (1950) have suggested that ox-liver β-glucuronidase is a single enzyme with a single pH optimum at 4·5, the type of pH-activity curve obtained in the present work using a highly purified enzyme preparation is suggestive of the complexity of the enzyme system. The most interesting feature in this respect is the observation that all highly purified enzyme preparations when allowed to stand for any length of time (3 weeks) showed a plateau-shaped curve similar to that illustrated by Sarkar & Sumner (1950) for their purified β-glucuronidase preparation. The pH-activity curve of an enzyme is the resultant of a number of factors, including enzyme stability, and it might be assumed that the latter factor plays a predominant part in determining the activity curve of purified glucuronidase preparations. Such preparations have low-protein contents, and the enzyme may be susceptible to pH changes in the absence of the protective protein. This possibility receives support from the observation that less pure

enzyme preparations, characterized by a lower activity/protein ratio, maintain the well defined optima on standing for periods of several weeks.

No evidence is forthcoming from the present work to show that ox-liver β-glucuronidase contains a coenzyme dissociating from the molecule on dilution. The discrepancy between the present findings and those of Bernfeld & Fishman (1950*b*) for calf-spleen β-glucuronidase may be due either to the widely differing methods of purification employed, or to the effect of dilution on the stability of the enzyme prepared by Bernfeld & Fishman (1950*b*). Our investigation of the effects of added DNA on liver glucuronidase activity emphasize the fact that this compound does not act as a coenzyme-like substance. While DNA is an effective activator of liver β-glucuronidase at pH 4·5, as observed by Bernfeld & Fishman (1950*b*), the activating effect at this pH can be reproduced by a variety of other agents such as albumin, gum acacia and starch. Since the completion of this work, Beyler & Szego (1952) have also reported the activating effect of albumin on glucuronidase activity.

Wills & Wormall (1950) and Wills (1952) have shown that many enzymes are inhibited by suramin in acid solution, but not in neutral or alkaline solution, the inhibition falling sharply at or about the isoelectric point of the enzyme. It was suggested by the above authors that suramin does not combine directly with the active centres of such enzymes, but that inactivation involves the combination with, or association of, the sulphonic acid groups of suramin with basic groups lying near, or perhaps on opposite sides of the active centre, with a resultant 'bridge' formation protecting the latter. While ox-liver β-glucuronidase falls into the group A enzyme classification of Wills & Wormall (1950), where a slight change of pH, itself without effect on the activity, has a profound effect on the degree of inhibition exerted by suramin, it is obvious that the bridging mechanism proposed for suramin action on such group A enzymes would not appear to be applicable in the case of glucuronidase. In the latter instance, small molecular weight acidic compounds can cause marked inhibition of enzyme activity.

The marked dependence on pH of inhibition by DNA, alginic acid, phthalic acid, 2:5-dihydroxyterephthalic acid, benzoic acid 3:5-disulphonic acid and 2-naphthol-3:6-disulphonic acid is compared with the suramin inhibition of β-glucuronidase in Figs. 7 and 8, and the fact that the curves fall sharply from 100% to zero inhibition over a very narrow pH range in all cases suggests that certain reservations should be made when applying the suramin inhibition method to a determination of the isoelectric point of certain enzymes.

The effect on β-glucuronidase activity of aromatic acids appears to conform to certain well-defined principles, namely: (1) The presence of one acid group on the benzene ring causes activation at all pH values studied. The sulphonic acid group appears to be more effective in this respect than the less strongly ionized carboxyl group. (2) Introduction of another acid group into the molecule invariably results in inhibition of enzyme activity at pH 3·4 concurrent with activation at pH 4·5 and 5·2. (3) Introduction of hydroxyl groups into the monoacidic aromatic molecule is specific in effect. Provided the position ortho to the acid group is unsubstituted, the activating effect of the acid decreases progressively with hydroxyl substitution. 3:4-Dihydroxybenzoic acid is without effect on enzyme activity, while 3:4:5-trihydroxybenzoic acid functions as an inhibitor at all pH values. (4) In all instances, introduction of an hydroxyl constituent ortho to an acidic component results in complete inhibition of enzyme activity at pH 3·4 at the concentration of inhibitors studied (10^{-2}M). Direct attachment of the acid group to the benzene nucleus is not an essential feature of this inhibition since 2:5-dihydroxyphenylalanine, where the hydroxyl group is positioned ortho to a side chain containing a terminal acidic grouping, acts in a similar manner.

Inhibition by phenylalanine and tyrosine is of interest in view of their occurrence *in vivo*. The relative concentrations of both amino-acids required for inhibition are high and, while tyrosine is the more effective in this respect, a concentration of 10^{-3}M is required to effect 10% inhibition.

From a consideration of the available dissociation constants of the compounds listed in Table 3, it would appear that the degree of dissociation of any one compound has little relevance to the measure of inhibition or activation at any one pH, and that such effects are dependent more on the specific configuration of the molecule.

SUMMARY

1. A method has been devised for the purification of ox-liver β-glucuronidase, using reactions with metallic ions and organic solvents. An 800-fold purification has been achieved by such means, with a 5% recovery of the enzyme.

2. The pH-activity curve of the purified material shows optima at 4·5, 5·2 and 6·3, and the possible complexity of the enzyme is indicated.

3. The isoelectric point of purified ox-liver β-glucuronidase by filter-paper electrophoresis was found to be pH 4·85.

4. No evidence is forthcoming from the present work to indicate that ox-liver β-glucuronidase contains a coenzyme dissociating from the molecule on dilution.

5. Details are given of the inhibition and activation of ox-liver β-glucuronidase by deoxyribonucleic

acid, suramin, alginic acid and a number of other organic acids, the exact effect in each case varying with pH.

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Unimolecular Films of Polymyxins A, B, D and E at the Air-water Interface

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The polymyxins consist of a group of five water-soluble basic polypeptide antibiotics possessing a specific action against Gram-negative bacteria; they are produced by many strains of the organism *Bacillus polymyxa*. Benedict & Langlykke (1947) first reported the antibacterial properties of cultures of this organism.

Chemical analysis of the hydrolysates of the antibiotics showed that a fatty acid, identified by Wilkinson (1949) as D-6-methyloctan-1-oic acid, was present in all the polymyxins. Amino-acid analyses demonstrated that polymyxin A contained L-threonine, L-α,γ-diaminobutyric acid and D-leucine (Catch, Jones & Wilkinson, 1948); polymyxin B contained the above amino-acids with D-phenylalanine in addition (Catch, Jones & Wilkinson 1949); polymyxin C differed from polymyxin B in not containing D-leucine; polymyxin D possessed the same amino-acids as polymyxin A but in addition D-serine (Jones, 1948); and polymyxin E had the same amino-acid composition as polymyxin A, but the intact antibiotics could be readily differentiated by paper chromatography (Jones, 1948).

The chemotherapeutic and pharmacological properties of the five polypeptides were studied by Brownlee, Bushby & Short (1952) and showed that the polymyxins A, C and D all caused severe proteinuria when administered parenterally in animals and man, this occurring even with the purest samples of the antibiotics. In this respect polymyxins B and E differed considerably since they exhibited far less nephrotoxic action.

Relatively little information has accumulated concerning the physical properties of the antibiotics. Jones (1948, 1949) has carried out chromatographic analyses of the antibiotics and has also studied their electrometric titration curves. Bell *et al.* (1949) have determined the molecular weight of polymyxin D by an osmometric method.

In the present communication, the investigation is described of the properties of unimolecular films of four of the polymyxins when spread at the air-water

interface on concentrated salt solutions. These experiments would be expected to provide information concerning the structure and intermolecular packing of the polypeptides. In addition, in order to determine if the nephrotoxic polypeptides possessed significantly different molecular weights compared with the non-toxic compounds, measurements have been carried out at low surface pressures to obtain an estimate of the molecular weights of the antibiotics. This method was first introduced by Guastalla (1939, 1942, 1943) who found that when proteins, fats and fatty acids were spread as unimolecular films at the air-water interface, they behaved as non-ideal two-dimensional gases at low surface pressures. He was able to estimate the molecular weights of several proteins by the use of a two-dimensional equation analogous to that employed for molecular weight determination from osmotic pressure data. This method has been subsequently extensively investigated by Bull (1945, 1946, 1947, 1950) and by Allan (1951) at the air-water interface, and at the liquid-liquid interface by Cheesman (1952). The method finds best application for substances having a 'surface molecular weight' not greater than 20 000, since as the molecular weight increases, the surface pressures produced by the film molecules decreases, and the accuracy of their measurement decreases correspondingly.

EXPERIMENTAL

Apparatus. In the study of the surface characteristics of the polymyxins at high surface concentrations a standard Langmuir-Adam type surface balance was used with a sensitivity of 0·2 dyne/cm. Simultaneous recordings of the surface potential changes produced by the presence of the film molecules were made with the electrometer circuit described by Few & Pethica (1952). The surface potential changes (ΔV) have been plotted throughout as ΔVA against A, i.e. as the product of the surface potential in millivolts times the area of the unimolecular film in sq.m./mg. The function ΔVA is directly proportional to the vertical component of the permanent dipole of the oriented monolayer molecules (Schulman & Hughes, 1932).

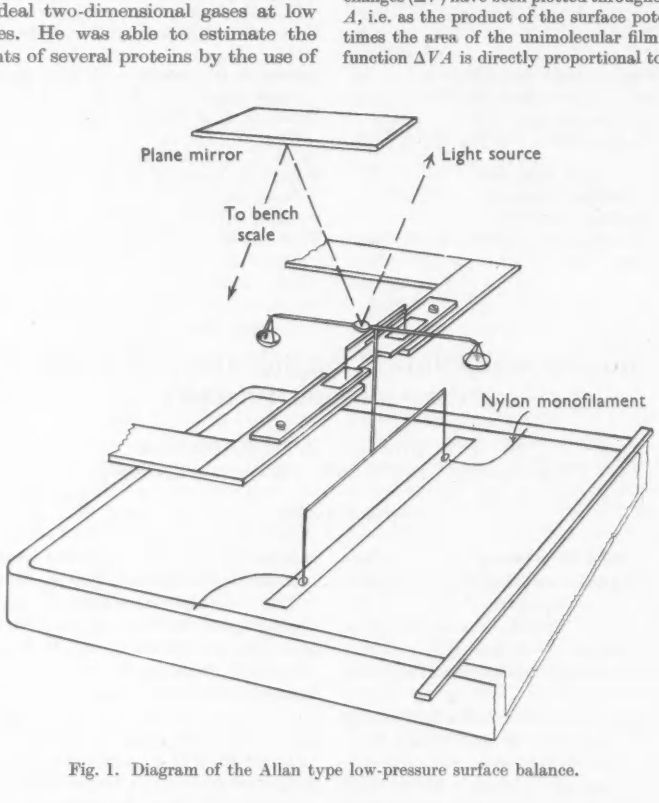

Fig. 1. Diagram of the Allan type low-pressure surface balance.

Of the two main methods available for the determination of surface pressures at high surface dilution, the Wilhelmy hanging-plate technique has been used by Bull (1945, 1946, 1947, 1950), Dieu & Bull (1949) and Cheesman (1952). However, Allan (1951) found that inconsistent results were obtained by this method, due mainly to contact angle changes at the Wilhelmy plate and to drifting of the zero reading caused by drainage and evaporation of the trough

solution from the plate. To overcome these difficulties a modified horizontal Alexander type (1947) film balance was developed by Allan (1951) and this balance has been used in the present investigation.

The low-pressure film balance is shown diagrammatically in Fig. 1. The torsion strip of the Alexander balance was removed and replaced by a horizontal steel strip, near the centre of which were cemented two microscope-slide coverslips. The movable suspension of the balance was fitted with two hardened steel screws ground to a point and adjusted to give a sensitive bearing, in the form of a balance bridge, when the suspension was placed in position on the glass coverslips. A paraffin-waxed mica float was made with two holes in it, through which passed freely two platinum wires attached to the balance bridge. This bridge had a mirror fitted in a horizontal position and two short arms each carrying small aluminium pans. Weights were added to these pans both for calibration and to adjust the bridge so that it rested horizontally with clean water in the trough. With this instrument the sensitivity was governed primarily by the threads attached to the mica float and used to confine the unimolecular film. Using Nylon monofilament threads of a diameter 0·125 mm., rendered hydrophobic by a thin film of petroleum jelly, and with an optical lever of 200 cm. to magnify the movement of the balance bridge, the instrument had a sensitivity between 0·3 and 0·4 cm./0·01 dyne/cm. surface pressure. The surface balance was contained in a wooden box with a removable Perspex front. In one side of the box a hole was made through which was fitted the barrel of an Agla micrometer syringe. The syringe was clamped rigidly in a horizontal position but was free to rotate about its cylindrical axis. A no. 6 serum hypodermic needle was bent at right angles and attached to the syringe; by rotating the syringe, the tip of the needle could be made just to touch the surface of the aqueous solution contained in the trough. Measured volumes of polypeptide solutions could thus be added to the aqueous surface with a minimum of disturbance of the instrument.

After thorough cleaning of the surface of the trough solution, the sensitivity of the balance was determined and the surface was again swept. Successive points on the force-area curves (*F*-*A*) were obtained at constant trough area by the addition of measured volumes of the polypeptide dissolved in a 0·05% (v/v) *iso*propanol-water solution. Two min. were allowed to elapse after each addition of the solution before recording the pressure. This time interval was sufficient to allow for the disappearance of the *iso*propanol-water solvent from the surface phase. Measurements were made at 20° ± 1.

Calculation of molecular weights from low surface pressure measurements. The equation of state of an ideal two-dimensional gas can be written in the form: $FA = RTW/M$ (Adam, 1926; Mitchell, 1935), where F is the surface pressure in dynes/cm., A is the area in sq.cm., W = weight of film spread in g., M is the molecular weight, R is the two-dimensional gas constant = $8{\cdot}31 \times 10^7$ erg/° K, and T the absolute temperature. With the assumption that this equation applies at zero surface pressure and when A is expressed in sq.m./mg. the molecular weight can be determined from

$$M = \frac{RT}{\lim_{F=0} FA} = \frac{2435}{\lim_{F=0} FA} \text{ at } 20°.$$

The value lim. FA is obtained by extrapolation of the FA-F plot to F = zero. This equation is analogous to the van't Hoff relation for osmotic pressure.

Materials. Polymyxins A, B and E were kindly supplied by the Wellcome Research Laboratories, Beckenham, Kent, and a sample of polymyxin D was supplied by the American Cyanamide Co., Stamford, Conn., U.S.A. These materials were used without further purification. Spreading solutions of the polypeptides were prepared in a 0·05% (v/v) *iso*propanol-water solvent at a concentration of about 0·2 mg./ml. Attempts to form stable monolayers of the polypeptides on 0·85% (w/v) NaCl at pH 7 and 10, and 15% (w/w) Na_2SO_4 at pH 7 were unsuccessful, as the polypeptides rapidly passed into the trough subphase on compression to a few dynes/cm. Stable monolayers were obtained by using 70% (w/v) $(NH_4)_2SO_4$, pH 4·5, as the trough solution and this subsolution has been used throughout the investigation. Surface active impurities in this solution, which was prepared using A.R. grade $(NH_4)_2SO_4$, were removed by the addition of 5 g. 'Karbac' activated carbon to 1 l. of solution, and after 3 hr. contact the carbon was removed by filtration. Three such treatments were found to give a solution free from surface active impurities.

RESULTS

Measurements of low surface pressures. The results obtained for low surface pressure measurements using polymyxins A, B, D and E showed that the polypeptides could be divided into two groups. In Fig. 2 are shown the points for determinations on

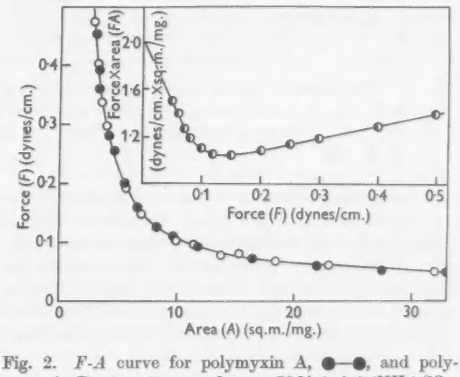

Fig. 2. F-A curve for polymyxin A, ●—●, and polymyxin D, ○—○, spread upon 70% (w/w) $(NH_4)_2SO_4$, pH 4·5, at 20°. Insert: FA-F curve for polymyxins A and D, ◐—◐.

polymyxins A and D. These two polypeptides gave identical force-area (*F*-*A*) curves over a pressure range of 0·05–0·5 dyne/cm. and a surface concentration range of 35–2·5 sq.m./mg. The insert FA-F curve in Fig. 2 was calculated using values read from the smoothed *F*-*A* curve. Extrapolation of the linear low pressure portion of this curve to $F = 0$ gives lim. $FA = 2{\cdot}0 \pm 0{\cdot}1$ and a molecular weight of

1220 ± 60 for these polypeptides. The experimental error of $\pm 5\%$ in the value lim. FA is primarily due to an error of approximately $\pm 2\%$ in the pressure readings below 0·1 dyne/cm.; the area measurement was obtained with an error of less than $\pm 1\%$. Fig. 3 shows the experimental points obtained for both polymyxins B and E. Again, within experimental error the results can be represented by a single F-A curve over a pressure range of 0·04–0·5 dyne/cm. and a surface concentration range of 31–2·0 sq.m./mg. The insert FA-F curve in Fig. 3 was calculated as in the previous case. Extrapolation of the linear low pressure portion of this curve to $F = 0$ gives lim. $FA = 1{\cdot}9 \pm 0{\cdot}1$ and the molecular weight 1280 ± 70. There is, therefore, no significant difference in the molecular weights of the four polypeptides as measured by this method.

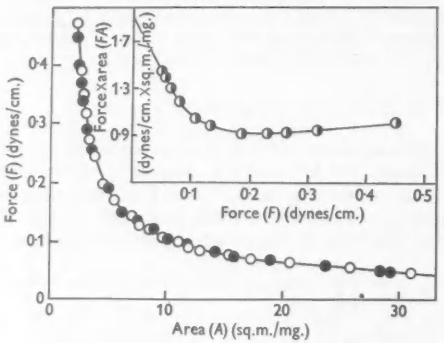

Fig. 3. F-A curve for polymyxin B, ●—●, and polymyxin E, ○—○, spread upon 70% (w/w) $(NH_4)_2SO_4$, pH 4·5, at 20°. Insert: FA-F curve for polymyxins B and E, ◑—◑.

Examination of the two F-A curves of the polymyxins shown in Figs. 2 and 3 indicates that at pressures below 0·15 dyne/cm. they exhibit very similar properties, whilst above this pressure the F-A curves become increasingly divergent. Thus at an area of 3 sq.m./mg. polymyxins A and D produce a pressure of 0·44 dyne/cm. and polymyxins B and E give only 0·31 dyne/cm.

A minimum occurs in each FA-F curve at a pressure of between 0·15 and 0·20 dyne/cm. These correspond to that observed by Bull (1947) for zein films at the air-water interface, and by Cheesman (1952) for bovine serum albumin at the benzene-water interface. Bull has suggested that the minimum observed with zein films may not be due to attractive forces operating between the molecules in the surface phase, but to the displacement of small and soluble fragments of the film molecules into the aqueous subsolution as the film is compressed. As 6-methyloctanoic acid is contained in all the polymyxins, we suggest that the minimum observed for these polypeptides is not due to a disruption of the molecule but is caused by van der Waals' forces of attraction between the adjacent C_8 hydrocarbon chains of the film molecules, the effect of which becomes less as the surface concentration decreases.

Measurement of high surface pressures. The results obtained with surface films of these four polypeptides at high surface concentration are recorded in Fig. 4. Polymyxins A and D produce identical F-A curves, characterized by a collapse pressure of 22·6 dynes/cm. and a minimum coefficient of compressibility ($-dA/AdF$) of 0·018 cm./dyne at an area of 0·85 sq.m./mg. Polymyxins B and E give significantly different F-A curves when compared with those of polymyxins A and D. Whilst polymyxin B appears to occupy a slightly smaller area than polymyxin E at all pressures, the curves are in other respects closely similar. Thus both the polypeptides have a collapse pressure of 31·3 dynes/cm. and have the same minimum coefficient of compressibility of 0·013 cm./dyne at 0·70 and 0·75 sq.m./mg. for polymyxins B and E respectively. The slightly smaller area occupied by polymyxin B compared with polymyxin E may be due to the dissolution into the trough solution of traces of impurities of low molecular weight present in the sample used, although the close correspondence of the low pressure F-A curve does not support this view.

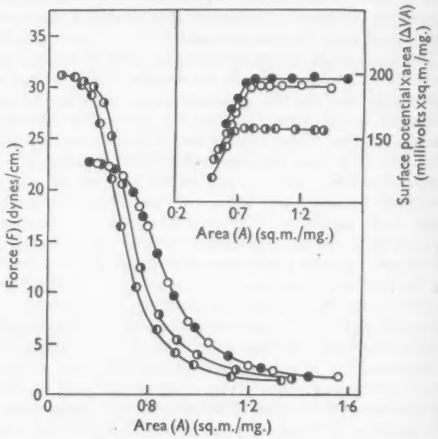

Fig. 4. F-A curves for polymyxin A, ○—○, polymyxin B, ◐—◐, polymyxin D, ●—●, and polymyxin E, ◑—◑, spread on 70% (w/w) $(NH_4)_2SO_4$, pH 4·5, at 20°. Insert: ΔVA-A curves for the polymyxins A, B, D and E. The symbols have the same significance as in the F-A curves.

The curves of ΔVA against A in Fig. 4 also fall into the same groups as in the case of the high pressure F-A curves. The function ΔVA is directly proportional to the vertical component of the dipolar molecules forming the film, and is constant at film areas greater than 0·9 sq.m./mg. for all the polypeptides. The constant ΔVA for polymyxins A and D is greater than that exhibited by polymyxins B and E. These constant values obtained for ΔVA as the films are compressed from large areas indicate that no molecular reorientation occurs during this stage. This holds until the films are compressed to the areas giving minimum compressibility. On further compression below these critical areas the values of ΔVA begin to decrease, showing that reorientation of the film molecules has commenced. It is interesting to note that Bull (1947) has suggested, for protein films, that the area at minimum compressibility represents the smallest area to which a film can be compressed without partial collapse taking place; pressure readings taken at areas below this critical area become much more uncertain and dependent upon time. In the case of the polypeptides investigated here, whilst a reorientation of the film molecules undoubtedly takes place, pressure readings taken at areas smaller than those giving minimum compressibility were quite stable and reproducible. The collapse pressures of the polypeptides were similarly more readily reproducible than those of protein films, and this may be due to the considerable difference in molecular size of the two classes of compounds.

The structure of the polypeptide monolayers as revealed qualitatively by the observation of the motion of talc particles placed on the film surface showed that the films were liquid over the whole compression range. At high surface pressures no evidence for a 'gel' structure was observed, such as obtained with protein films (Hughes & Rideal, 1932); this might be due to a reduction of intermolecular hydrogen bonding in the presence of trough subsolution of low pH as discussed by Alexander (1942).

DISCUSSION

The results obtained for the molecular weights of polymyxins A, B, D and E based upon low surface pressure measurements with insoluble monolayers of the polypeptides show that they all possess the same surface molecular weight within the limits of experimental error. The mean molecular weight 1250 is in good agreement with the value 1150 found by Bell *et al.* (1949) for polymyxin D. It would indicate the presence in the four polypeptides of about nine amino-acid residues in addition to the fatty acid component as suggested by Bell *et al.* (1949) for polymyxin D. These authors also concluded that the various biologically active materials related to polymyxin D were probably closely related in composition, as confirmed in this investigation.

Although there appears to be little significant difference in the molecular weights of the polymyxins, the characteristics of their films at high surface concentration show that the four polypeptides can be classified in two groups according to the compressibilities, collapse pressures and electrical properties of their monolayers. In these respects, polymyxins A and D have identical values, whilst films of polymyxin B appear to be slightly more closely packed than those of polymyxin E, but otherwise have identical properties. The polymyxins in these two groups have different amino-acid constitutions, but the monolayer results indicate that within both groups the intramolecular orientation of the amino-acids and fatty acid residues are closely similar. Moreover, under conditions of equal surface pressure the molecules of the polymyxins A and D are not capable of such close surface packing as those of polymyxins B and E. This, together with the greater compressibility of the polymyxins A and D monolayers, suggests that the intramolecular structures of these polypeptides are not as compact as those of polymyxins B and E.

It is interesting to note that polymyxins A and D, in addition to having identical monolayer properties, also possess similar pharmacological action since both these antibiotics produce severe proteinuria in animals, whereas polymyxins B and E produce only slight toxic effects (Brownlee *et al.* 1952).

SUMMARY

1. The properties of monomolecular films of polymyxins A, B, D and E have been investigated at air-water interface.

2. The molecular weights of the four polypeptides have been determined by application of a two-dimensional gas law equation to measurements carried out at low surface concentration. These results show that there is no significant difference in the molecular weights of the antibiotics.

3. Studies at high surface concentration have shown that the four polypeptides can be separated into two groups, polymyxins A and D in one group and polymyxins B and E in the other. The classification depends upon the compressibilities, collapse pressures and electrical properties of their monolayers. These results have been discussed with regard to the known pharmacological action of the polypeptides.

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